# Substance P and Neurokinin A as Markers of Pain in Neonates

C Mae Wong

B.Med.Sci.(1st), B.M.B.S., M.R.C.P.(U.K.), M.R.C.P.C.H.

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# Declaration

I hereby declare that this thesis is my own composition. The laboratory experiments, study design, sample collection, sample analyses, data analyses, and dissemination of results have been carried out predominantly by myself, as part of a research team. Contributions from co-workers have been acknowledged. No part of this work has been submitted for any other degree.

Department of Child Life and Health

University of Edinburgh

20 Sylvan Place

Edinburgh EH9 1UW

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## Abstract

The recognition and treatment of pain is a constant challenge in the care of newborn infants. Various behavioural and physiological measures exist, but mainly for acute pain. They are also less applicable in the sickest and most premature infants. Most existing neurochemical measures are markers for stress rather than pain. Both substance P (SP) and neurokinin A (NKA) have been postulated to be involved in persistent pain in adult studies, but never previously researched in newborn infants.

Methods of sample handling and analysis were developed to accommodate neonatal microsamples. Sample extraction was found to be essential, as were the speed of sample collection and the use of polypropylene materials during sample handling.

The clinical study enrolled 174 infants of different gestational ages, who had serial measurements of SP, NKA and cortisol performed in plasma and saliva samples. Plasma SP concentrations in neonates ranged from 0.0-11.2 pmol/L (median 1.7 pmol/L) and NKA concentrations from 1.8-74.6 pmol/L (median 6.0 pmol/L). Gestation and birth weight had no significant correlation with peptide concentrations. Postnatally, there was a gradual rise in median plasma SP and NKA during the first three days which decreased again by days 7 to 14. Perinatal factors such as labour, the mode of delivery, and epidural analgesia affected NKA but not SP concentrations.

With regard to either pain or assisted ventilation, plasma SP concentrations did not appear to be a useful marker of persistent pain or distress. Conversely, plasma NKA concentrations showed significant changes with ventilation, which were further

modulated by the use of analgesia. Cortisol responses in the same group of infants demonstrated significant changes with ventilation, but not with the administration of analgesia. This suggests that although cortisol is a useful indicator of overall stress, NKA might be more specific for pain.

There was a weak correlation between plasma SP and NKA. Plasma SP did not correlate with plasma cortisol or other physiological measures of pain used in this study. Plasma NKA did not correlate with plasma cortisol, but showed some correlation with behavioural pain scores. Neither neuropeptide correlated with heart rate variability, whereas plasma cortisol did. As with plasma cortisol, heart rate variability was not modulated by the use of analgesia, suggesting that both were markers for stress but not necessarily for pain.

Worldwide, this is the first study of SP and NKA in newborn infants. Assessing pain in neonates is a challenge, yet pain in neonates has detrimental consequences and needs to be treated appropriately. The ability to neurochemically measure pain in infants undergoing neonatal intensive care, as well as monitor responses to analgesia, might one day facilitate more efficient pain management.

# Abbreviations

| | |
|---|---|
| ACE | angiotensin-converting enzyme |
| ACN | acetonitrile |
| ACTH | adrenocorticotrophic hormone or adrenocorticotrophin |
| ANOVA | analysis of variance |
| AUC | area under the curve |
| cAMP | cyclic adenosine monophosphate |
| CGRP | calcitonin gene-related peptide |
| $cmH_2O$ | centimetres of water |
| cpm | counts per minute |
| CS | caesarian section |
| CSF | cerebrospinal fluid |
| DARS/NRS | donkey anti-rabbit serum with normal rabbit serum |
| DSVNI | Distress Scale for Ventilated Newborn Infants |
| ECG | electrocardiograph |
| EDTA | ethylenediaminetetraacetic acid |
| EFIC | European Federation of the IASP Chapters |
| FDA | Food and Drug Administration |
| FSH | follicle-stimulating hormone |
| GABA | gamma-aminobutyric acid |
| GDNF | glial cell-derived neurotrophic factor |
| HPLC | high-performance liquid chromatography |
| Hz | Hertz |
| IASP | International Association for the Study of Pain |

| | |
|---|---|
| IB4 | isolectin B4 |
| IBCS | Infant Body Coding System |
| IVH | intraventricular haemorrhage |
| LH | luteinising hormone |
| LIDS | Liverpool Infant Distress Scale |
| LPE | liquid phase extraction |
| LPS | lipopolysaccharide |
| µl | microlitre |
| µV | microvolt |
| MBPS | Modified Behavioural Pain Scale |
| mmHg | millimetres of mercury |
| mRNA | messenger ribonucleic acid |
| ms | millisecond |
| MSH | melanocyte-stimulating hormone |
| mV | millivolt |
| NAPI | Neonatal Assessment of Pain Inventory |
| NEC | necrotising enterocolitis |
| NEOPAIN | Neurological Outcomes and Pre-emptive Analgesia in Neonates |
| NEP | neutral endopeptidase |
| NFCS | Neonatal Facial Coding System |
| NHS | National Health Service |
| NIPS | Neonatal Infant Pain Scale |
| NKA | neurokinin A |
| NKB | neurokinin B |

| | |
|---|---|
| NK1R | neurokinin-1 receptor |
| nmol/L | nanomols per litre |
| Ω | Ohm |
| PAT | Pain Assessment Tool |
| PEG | polyethylene glycol |
| PIPP | Premature Infant Pain Profile |
| pmol/L | picomoles per litre |
| PPT-A | preprotachykinin-A |
| SD | standard deviation |
| SIDS | sudden infant death syndrome |
| SP | substance P |
| SPE | solid phase extraction |
| SUN | Scale for Use in Newborns |
| SVD | spontaneous vertex delivery |
| TFA | trifluoroacetic acid |
| TrkA | tyrosine kinase receptor A |
| TSH | thyroid-stimulating hormone or thyrotrophin |
| VIP | vasoactive intestinal peptide |
| WHO | World Health Organisation |
| %B/Tot | percentage bound over total |
| %B/Ref | percentage bound over reference |

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# 1. GENERAL INTRODUCTION

## 1.1 THE NATURE OF PAIN/WHAT IS PAIN?

"Pain is perfect misery, the worst of evils, and, excessive, overturns all patience."

Milton. *Paradise Lost.*

Pain has been defined by the International Association for the Study of Pain (IASP) (www.iasp-pain.org) as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage… Pain is always subjective. Each individual learns the application of the word through experiences related to injury in early life"[1]. In contrast, the noxious perception of pain without taking into account its emotional aspect is referred to as nociception.

Pain experience, therefore, involves psychological and emotional variables, and can markedly differ between individuals, depending on previous pain experience, biological variation, psychological aspects, and even cultural factors. There can be a lack of correspondence between the extent of injury and the intensity of pain experience. One difficulty with the definition of pain lies with the statement that pain requires learning and previous experience. This would render it impossible for a fetus or newborn to experience pain. Yet there is much evidence to prove that suffering pain does not require previous experience (See Section 1.2). Another problem with this definition is that infants have a limited ability to communicate pain. Fortunately, the IASP has more recently noted that "the inability to communicate verbally does

not negate the possibility that an individual is experiencing pain and is in need of appropriate pain-relieving treatment."[2]

Other definitions of pain-related terms include:

Acute pain: Pain associated with a brief episode of tissue injury or inflammation

Chronic pain: Constant pain over a period of three months or longer

Hyperalgesia: An increased response to a stimulus which is normally painful

Allodynia: Pain due to a stimulus which does not normally provoke pain

Neurogenic pain: Pain initiated or caused by a primary lesion, dysfunction, or transitory perturbation in the peripheral or central nervous system

### 1.1.1 The function of pain

The pain experience serves to inform the sufferer of potential or existing tissue injury. Patients who lack pain sensation are vulnerable to more extensive trauma, as they are deficient in a self-protective mechanism. As such, pain in an individual can also serve to warn others about a potential threat to safety.

### 1.1.2 Pain fibres

Sensory nerve terminals that are activated during a painful stimulus include low threshold Aβ mechanoreceptors, and nociceptive Aδ and C fibres. Aδ fibres are thinly myelinated high threshold mechanoreceptors, while C fibres are unmyelinated polymodal nociceptors. Nociceptors are triggered by a variety of noxious stimuli, including low extracellular pH, heat, and capsaicin (which is derived from hot chilli peppers). C fibres are divided into two groups: those that contain neuropeptides (e.g.

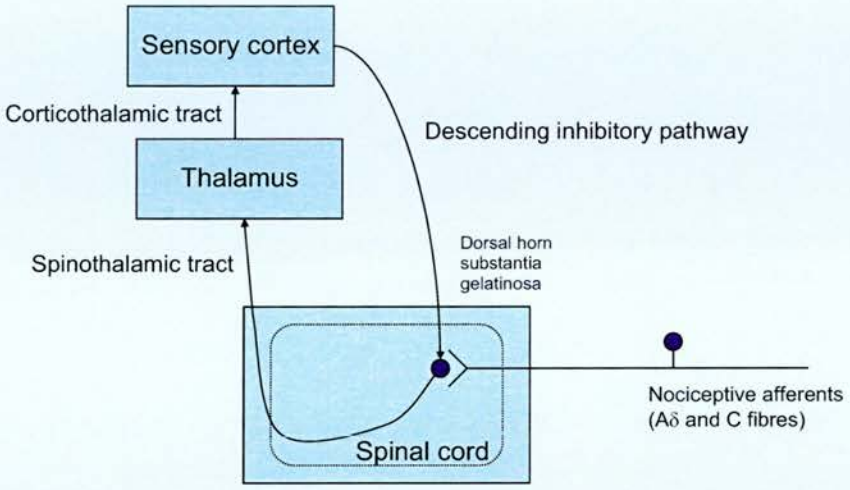
substance P or SP) and express the neurotrophin receptor for nerve growth factor, and those which bind isolectin B4 (IB4) and express receptors for the neurotrophin glial cell-derived neurotrophic factor (GDNF). C fibres terminate in the Rexed's lamina II of the substantia gelatinosa of the dorsal horn. This is a site pain research has focused on, rich in neuropeptide and enzyme activity, and thought to be an area of modulation[3].

In adults, lamina II of the substantia gelatinosa of the dorsal horn is exclusively occupied by C fibres, but in neonatal rat pups, it is also occupied by transient A fibre terminals. These may be pressure-sensitive receptors, and there is some evidence that rapid nociceptive responses are mediated via these fibres in the newborn[4].

### 1.1.3 Pain pathways

A diagrammatic representation of the neuronal pathways involved in pain signal transmission and modulation is shown in Figure 1.1.

**Figure 1.1:** Pain pathways




### 1.1.4 Nociceptor differentiation

Nociceptors are specified early in development, even before they form contacts with their future innervation targets[4]. In the dorsal root ganglia, the large-diameter A fibres are born before the small-diameter C ones. The small-diameter fibres express tyrosine kinase receptor A (TrkA) and appear to be under separate transcriptional control to the large-diameter fibres, which express tyrosine kinase receptors B and C. Around the time of birth in rat pups, a subpopulation of nociceptors downregulates the expression of TrkA and becomes the IB4-positive nociceptors. The rest continue to differentiate depending on neurotrophin levels during the postnatal period, and also peripheral target innervation. Subsequently, the innervation density of nociceptors in the skin is regulated by local neurotrophins, and can be upregulated by neonatal skin wounding which increases local neurotrophin levels, resulting in hyperinnervation of the skin[5].

Centrally, lamina I projection neurones are complete before the generation of local circuit modulatory neurones, indicating that nociceptive transmission from the spinal cord to higher centres could develop before local modulation is ready[4]. At premature birth, a noxious stimulus peripherally results in a whole-body untuned movement response, which only becomes a more specific limb withdrawal with ongoing development. Cutaneous reflexes have lower thresholds and result in a more prolonged muscle contraction response in prematurity. Local modulation subsequently occurs via GABA-mediation initially, and via glycinergic synapses later on in postnatal life. Central modulation via descending fibres which contain serotonin also takes place even later as there is prolonged postnatal maturation of the descending pathways.

## 1.2 Evidence for pain in neonates

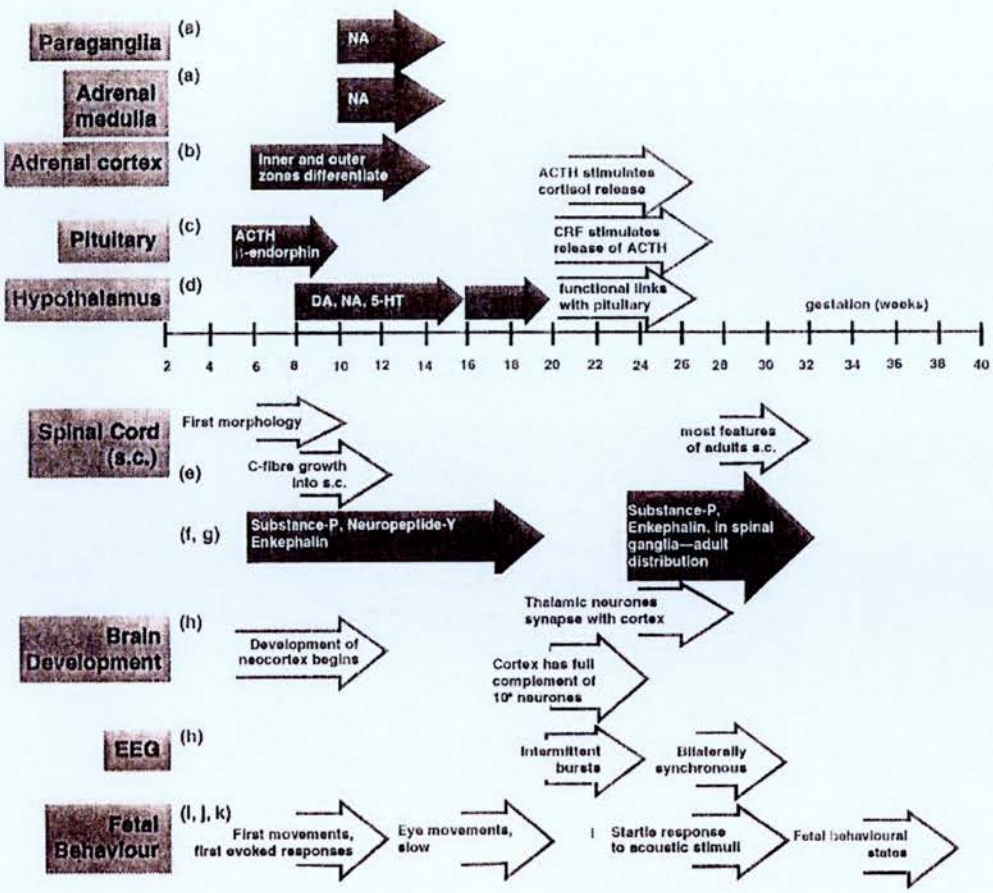
Pain in infants was largely ignored till the late 1960s. This was due to a combination of misconceptions within the medical and nursing professions: firstly, that infants had a low perception of pain; secondly, that they would be highly susceptible to adverse reactions from analgesia and anaesthesia; and thirdly, that there was no need to treat their pain as they would have no memory of it. For example, infants would undergo a thoracotomy for patent ductus arteriosus ligation using only nitrous oxide and tubocurarine.

How then do we know that human neonates feel pain?

### 1.2.1 Anatomical evidence

The neuroanatomical requirements for pain perception are present even in the most preterm infants. Figure 1.2 summarises the fetal development of various components of the nervous system.

**Figure 1.2:** Development of some aspects of the fetal hypothalamic-pituitary axis, central nervous system and behaviour




From Glover V and Giannakoulopoulos X. Stress and Pain in the Fetus. Baillieres Clinical Paediatrics, 1995.

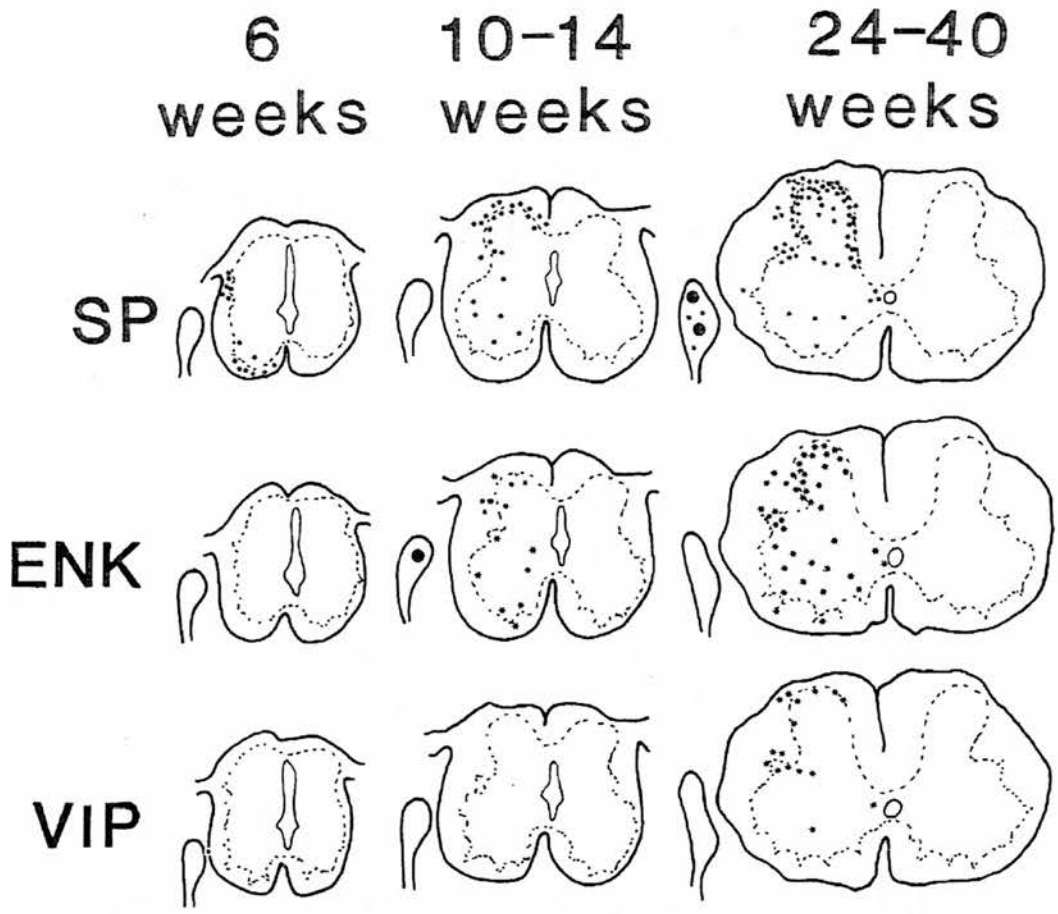
*1.2.1.1 Neuronal distribution*

In the human embryo, both A and C dorsal root ganglion cells are formed by 5 weeks of development. The sensory neurones that give rise to nociceptors grow out from the dorsal root ganglion during embryo development, and proceed to innervate the skin in an organised proximodistal manner[6]. Large Aβ fibres develop by 8 weeks[7], while C fibres develop later. Innervation of the perioral area occurs by 7 weeks, the face and hands by 11 weeks, trunk and legs by 15 weeks. By 20 weeks in humans, these neurones reach the most distal skin of the foot[8]. The cortex has its full complement of $10^9$ neurones by 20 weeks' gestation, and corticothalamic connections are complete by 24 weeks' gestation. Further growth of synaptic and dendritic formations then continues to occur during fetal and postnatal development.

*1.2.1.2 Neuropeptide distribution*

Pain-related neurotransmitters or neuromodulators in the spinal cord, including SP, enkephalin, somatostatin, calcitonin gene-related peptide (CGRP), vasoactive intestinal peptide (VIP), and other ligands, seem to follow similar developmental patterns of expression in newborn rats and human neonates (Figure 1.3)[9]. Neurotransmitters act via synaptic receptors to produce a functional response, whilst neuromodulators modify the state of the receptor or second messenger to facilitate or reduce the action of a neurotransmitter. They are both present in Aδ and C afferents, and all are expressed before birth in the human spinal cord. By 8-10 weeks' gestation, SP, somatostatin and CGRP are present in the spinal cord, with enkephalin and VIP appearing later. A further increase in peptide concentrations occurs at 28-30 weeks[10;11], and again at birth.

**Figure 1.3:** The distribution of substance P, enkephalin and vasoactive intestinal peptide in the developing human spinal cord




From Marti E, et al. Ontogeny of peptide- and amine-containing neurones in motor, sensory, and autonomic regions of rat and human spinal cord, dorsal root ganglia, and rat skin. *J.Comp Neurol.* 1987;**266**:332-59.

### 1.2.2 Physiological evidence

As with adults, activation of the sympathetic nervous system in response to pain produces physiological changes characteristic of the 'fight or flight' state. Heart rate,

blood pressure, and respiratory rate increase (See Section 1.4.2). There are also changes in oxygen saturation, vagal tone, pupil dilatation, and palmar sweating.

### 1.2.3 Behavioural evidence

To the parents or carers, the most instantly obvious indication of acute pain in an infant would be behavioural changes, such as crying, and facial and body reactions (See Section 1.4.1). In persistent or chronic pain, behavioural changes may be more subtle, e.g. disruption of sleep, feeding, and perhaps more unexpectedly, an unduly quiet demeanour.

### 1.2.4 The memory

Although conscious memories of pain in early life may not be apparent, the human body has its own inherent subconscious memory. It has been demonstrated that infants develop an increased sensitivity to pain in later life, if exposed to unpleasant stimuli as a newborn[12]. This 'memory' was described by Taddio *et al* in two studies of pain reactions during subsequent routine vaccination of infants who had been circumcised as newborns[13;14]. Compared with non-circumcised males, circumcised males displayed higher behavioural pain scores and cried longer. In the second study, the use of a local anaesthetic cream during circumcision attenuated this subsequent exaggerated pain response[14].

Animal research has supported this with the finding that lack of analgesia provision results in an alteration of the neuronal pathways responsible for pain transmission in the spinal cord, and a consequent hyperalgesia[15]. Anatomical changes such as

abnormal sprouting of primary afferents, and hyperinnervation of cutaneous tissue, are seen following tissue injury[16-18]. Rats who experienced neonatal pain had lower pain thresholds, defensive withdrawal behaviour, and hypervigilance during adulthood[18;19].

## 1.3 IMPORTANCE OF PAIN IN NEONATES

### 1.3.1 Physiological and clinical evidence – the stress response

The stress response is the term used to describe the hormonal and metabolic changes that follow physical or psychological trauma. This may or may not be associated with the experience of pain. Stress hormones, such as adrenaline, noradrenaline, cortisol, glucagon, aldosterone, thyroid-stimulating hormone, and growth hormone, encourage catabolic activity and water retention. They simultaneously increase heart rate, blood pressure, and cardiac output, and increase the body's metabolic rate. They also cause hyperglycaemia by promoting the release of glucose from glycogen and preventing its utilisation. Gastric and bowel function and motility are also impaired. Lastly, stress hormones also impair immune function and are pro-coagulant.

Anand *et al* first showed in 1985 that neonates had the ability to mount an endocrine and metabolic stress response to surgery[20;21]. In a seminal study in 1987, Anand *et al* then proceeded to prove that this neonatal stress response was related to the undertreatment of pain[22]. This randomised controlled trial showed that major hormonal responses were significantly greater in infants who received only nitrous oxide and tubocurarine for patent ductus arteriosus ligation, compared with those who received fentanyl. The clinical importance of this was demonstrated in another

randomised controlled trial in 1992, where neonates receiving deeper anaesthesia were found to have a lower incidence of sepsis, metabolic acidosis, disseminated intravascular coagulation and death[23]. Untreated pain therefore exacerbates tissue injury, prevents healing, increases susceptibility to infection, prolongs hospitalisation and increases the risk of mortality.

### 1.3.2 Long-term impact on pain experience

Tied in with the body's physical memory of pain are the anatomical and behavioural changes which take place when a neonate, particularly if premature, experiences pain. As described in Section 1.2.4, even a single painful event has been shown to alter subsequent pain behaviour[13;14]. Repetitive painful stimuli can result in a 'rewiring' of pain pathways resulting in subsequent hyperalgesia or even allodynia. Premature infants are more vulnerable to this, and ironically, they are more exposed to multiple painful procedures in early life.

### 1.3.3 Compassion

Pathological consequences aside, the treatment and alleviation of pain are a basic human right that should take place whatever the age of the sufferer[24]. This was recently advocated in October 2004 by the IASP and the European Federation of the IASP Chapters (EFIC), with support from the World Health Organisation (WHO) (www.who.int)[25]. The statement was made during a one-day conference in Geneva. It coincided with new recommendations by the Council of Europe in Strasbourg on pain relief. One person in five suffers from moderate to serious chronic pain, and one in three of these are unable or only with difficulty able to lead an independent life.

Chronic pain is a major threat to the quality of life worldwide, and will become more so as the average age increases.

## 1.4 ASSESSMENT OF PAIN IN NEONATES

Quantifying pain in neonates is a challenge because they cannot verbally communicate this subjective phenomenon. This inability to communicate pain largely explains why healthcare professionals failed to recognise and treat pain appropriately, and continue to fail to do so. In the last few decades, however, various methods for identifying pain in neonates have been developed, though none on its own proves adequate. More recently, attempts to improve neonatal pain assessment have moved towards multi-dimensional scoring, involving a combination of behavioural and physiological factors.

### 1.4.1 Behavioural measures

Behavioural measures represent the current mainstay of pain assessment in neonates and there are many tools available (Table 1.1). They mainly comprise observations of body movement, facial expression, and cry. In selecting a pain assessment tool, the clinician must be aware that some tools have preliminary validity for infants of certain age groups only, or are designed for use with specifically procedural or post-operative pain, or even for use only in ventilated infants (e.g. DSVNI[26]).

One difficulty with behavioural measures is that infants who are more ill or premature may respond less vigorously to pain[27]. Although the behavioural response to acute pain is easily recognised, features of chronic or persistent pain may be

altogether different. Furthermore, medical treatment of the most severely ill infants may warrant the use of a paralysing agent. The elimination of all voluntary movement then precludes any behavioural assessment of pain.

**Table 1.1:** Clinical assessment of infant pain

| Measure and author | Age level | Indicators | Pain stimulus |
|---|---|---|---|
| Behavioral Pain Score *Pokela*[28] | Preterm and full term neonates | Facial expression, body movements, response to handling/consolability, rigidity of limbs | Procedural pain in ventilated neonates |
| Clinical Scoring System | Infants 1 to 7 months | Sleep facial expression, cry motor activity, excitability, digit flexion, sucking, tone, consolability, sociability | Postoperative pain |
| CRIES *Krechel & Bildner*[29] | Full term neonates | Crying, oxygen saturation, heart rate, blood pressure, expression, sleeplessness | Postoperative pain |
| Distress Scale for Ventilated Newborn Infants (DSVNI) *Sparshott*[26] | Preterm and full term neonates | Facial expression, body movement, color, heart rate, blood pressure, oxygen saturation | Procedural pain in ventilated neonates |
| Neonatal Facial Coding System (NFCS) *Grunau et al*[30] | Preterm and full term neonates, infants up to 4 months | Facial muscle group movement: brow bulge, eye squeeze, nasolabial furrow, open lips, stretch mouth, lip purse, taut tongue, chin quiver | Procedural pain |
| Infant Body Coding System (IBCS) *Craig et al*[31] | Preterm and full term neonates | Hand, foot, arm, leg, head, torso motor activity | Procedural pain |
| Liverpool Infant Distress Scale (LIDS) *Horgan & Choonera*[32] | Full term infants | Spontaneous movements, spontaneous excitability, flexion of fingers and toes, facial expression, quantity of crying, quality of crying, sleep | Postoperative pain |
| Modified Behavioural Pain Scale (MBPS) *Taddio et al*[33] | Infants 2 to 4 months of age | Facial expression, cry, gross motor movement | Immunization pain |
| Neonatal Assessment of Pain Inventory (NAPI) *Joyce et al*[34] | Infants 1 to 36 months | Smiling, sleeping, response to touch, sleeping, crying, respirations | Postoperative pain |

| Measure and author | Age level | Indicators | Pain stimulus |
|---|---|---|---|
| Neonatal Infant Pain Scale (NIPS) *Lawrence et al*[35] | Preterm and full term neonates | Facial expression, cry, breathing patterns, arms, legs, state of arousal | Procedural pain |
| Pain Assessment Tool (PAT) *Hodgkinson et al*[36] | Full term infants | Posture/tone, sleep pattern, expression, color, cry, respirations, heart rate, oxygen saturation, blood pressure, nurses' perception of infant pain | Postoperative pain |
| Premature Infant Pain Profile (PIPP) *Stevens et al*[37] | Preterm and full term neonates | Gestational age, behavioral state, heart rate, oxygen saturation, brow bulge, eye squeeze, nasolabial furrow | Procedural pain |
| Scale for Use in Newborns (SUN) *Blauer & Gerstmann*[38] | Preterm and full term neonates | Movement, tone, facial expression, behavioral state, breathing, heart rate, blood pressure | Procedural pain |

From Franck LS, Greenberg CS, Stevens B. Pain assessment in infants and children. *Pediatric Clinics of North America* 2000;**47**:487-512.

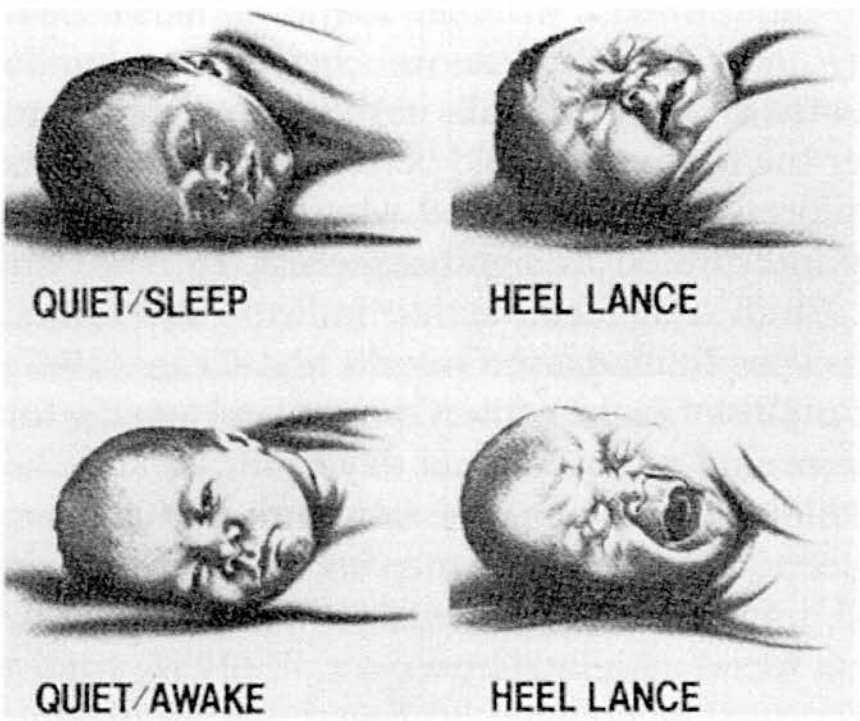
*1.4.1.1 Body movement*

An obvious reflex body reaction to pain is withdrawal, which occurs at the spinal level[39]. Other signs of pain include limb flailing, back and neck arching, and clenched fists. Body movement is relatively indiscriminate and a signal of overall distress rather than definite pain. If the source of pain is easily identifiable, then measurement of overall distress may be a better indicator of the impact of the pain experience. However if the source of pain is unclear, then global distress may confuse the assessment of pain[40].

*1.4.1.2 Facial expression*

The facial expression of pain is more specific and reliable than body movement[41]. It is displayed as a grimace, which comprises a furrowed brow, eye squeeze, a deepened nasolabial furrow, taut mouth, and cupped tongue (Figure 1.4). The most sensitive indicators are the first three, and the most specific the last two. Using these indicators, systematic measures of pain have been developed, such as the Neonatal Facial Coding System[30]. Other pain assessment tools exist that incorporate facial activity in their scoring (Table 1.1). However, in preterm infants with persistent pain, classic descriptions of frozen facial appearance and behaviour may not be as obvious[42].

**Figure 1.4:** The facial expression of pain



*1.4.1.3 Cry*

Crying is an important form of communication for the infant. It serves to alert the infant's carer of an infant's needs. It commonly accompanies a painful event but, as a marker of pain, is not 100% sensitive and is even less specific. Some infants do not cry following a painful incident, and crying is also used to indicate hunger and other non-painful distress. Studies have attempted to delineate a particular 'pain cry' using spectral analysis[27], and duration of cry has been used as a measure of analgesia[43-47].

As with other behavioural measures, cry is of limited use in the more severely ill infants. Not only are they less likely to produce a cry[27], but interventions such as endotracheal intubation make it very difficult to assess crying. If cry is used, healthcare professionals need to adjust their threshold for identifying pain based on it, depending on the infant's severity of illness.

*1.4.1.4 The Premature Infant Pain Profile*

This infant pain assessment tool is the most widely validated in both preterm and full-term infants for procedural pain[37;48]. It is a seven-indicator measure that comprises behavioural, physiological, and contextual indicators (Table 1.2). The PIPP has mainly been used to evaluate procedural pain, but has now been validated for evaluating post-operative pain[49].

**Table 1.2:** The Premature Infant Pain Profile (PIPP)

| Procedure | Indicator | 0 | 1 | 2 | 3 | Score |
|---|---|---|---|---|---|---|
| Observe infant for 15 sec<br>Baseline:<br>HR .....<br>$SaO_2$ .....<br>RR ..... | Gestational age (weeks) | > 36 | 32 to 35+6 | 28 to 31+6 | < 28 | |
| | Behavioural state | Active/awake<br>Eyes open<br>Facial movements<br>Crying | Quiet awake<br>Eyes open<br>No facial movements | Active sleep<br>Eyes closed<br>Facial movements | Quiet sleep<br>Eyes closed<br>No facial movements | |
| Observe infant for 30 sec | Heart rate increase<br>Max ..... | 0 – 4 bpm | 5 – 14 bpm | 15 – 24 bpm | > 25 bpm | |
| | $SaO_2$ decrease<br>Min ..... | 0 – 2.4% | 2.5 – 4.9% | 5.0 – 7.4% | > 7.5% | |
| | Brow bulge | None<br>0 – 9% of time | Minimum<br>10 – 39% of time | Moderate<br>40 – 69% of time | Maximum<br>> 70% of time | |
| | Eye squeeze | None | Minimum | Moderate | Maximum | |
| | Nasolabial furrow | None | Minimum | Moderate | Maximum | |
| **TOTAL SCORE** | | | | | | |

From Stevens B, Johnston C, Petryshen P, Taddio A. Premature Infant Pain Profile: development and initial validation. *Clin J Pain* 1996;**12**(1):13-22, with permission

### 1.4.2 Physiological measures

Pain initiates the stress response which produces quantifiable changes in physiological parameters. The same response may however be instigated by non-painful stressors. Furthermore these changes in physiological parameters may not be so apparent in chronic or persistent pain. Physiological measures do form part of some infant pain assessment tools (Table 1.1).

#### *1.4.2.1 Heart rate and heart rate variability*

In term neonates, the resting heart rate is generally between 100 to 140 beats per minute. A rise in heart rate is a well-recognised physiological response to a painful event, even in the most preterm infant. Processes that modulate cardiovascular function are closely related to those involved with pain reactivity[50;51]. Heart rate is controlled via a continuous feedback system which maintains blood pressure and central venous volume. This complex procedure involves the central nervous system, the autonomic nervous system, metabolic activity, and peripheral chemoreceptors and baroreceptors. States of arousal and physical activity also play a role. Hence heart rate is rarely constant, and heart rate variability represents continuous fine-tuning within the feedback system. Greater fluctuations in heart rate are thought to signify a healthier individual having a better ability to respond to changing environmental demands[52]. At rest, both parasympathetic and sympathetic influences are tonically active, with parasympathetic effects predominating. Parasympathetic output to the heart is linked rhythmically with the respiratory cycle, giving rise to respiratory sinus arrhythmia. There is a transient increase in heart rate with each inspiration, and subsequent decrease with expiration.

Heart rate variability can be analysed using various approaches, two common ones being in the time domain and in the frequency domain. An electrocardiogram signal is first obtained, then intervals containing ectopic beats or artefact are discarded. From the remaining normal beat series, the RR interval, or beat-to-beat interval, is measured. Heart rate is then expressed as beats per minute, or heart period. Heart rate variability can then be calculated either in the time domain or the frequency domain. A heart rate time domain signal can provide information on successive interval differences, in terms of their mean, standard deviation, or variance. In the frequency domain, spectral analysis can be used to quantify heart rate variability. Variability depends on the length of the selected heart rate epoch, increasing as the epoch increases[53], making cross-study comparisons difficult. Measuring heart rate variability in this manner is labour-intensive and mainly provides retrospective information, thus limiting its use to research.

A proxy measure of heart rate variability can also be obtained from cotside monitoring systems. Typically, the analysis of consecutive RR intervals takes place automatically, and a second-by-second rolling average heart rate is displayed. Variability is then calculated as the standard deviation of the heart rate[54]. Artefact is handled by routinely discarding a small percentage of the highest and lowest RR interval measurements. As this is performed universally, it results in some loss of relevant variability and is therefore not as accurate as the gold standard. However, it represents a more clinically applicable tool.

Heart rate is relatively easy and inexpensive to measure, and hence can be a useful adjunct in pain assessment. When used alone it can be difficult to interpret, as it is confounded by numerous physiological, behavioural and developmental factors, as well as illness, and medical or pharmacological treatment. For example, mean resting heart rate in term infants increases during the first two months of life, then decreases through subsequent infancy[55;56]. Heart rate variability does the opposite[57;58]. Preterm infants have a higher mean resting heart rate than term infants, and heart rate variability increases with postnatal age, but is lower at term compared with term-born infants[52;59;60]. This may suggest maturational delay. Small for gestational age term infants also have a higher heart rate compared to those appropriate for gestational age[61]. Mechanical ventilation and respiratory distress syndrome affect the heart rate frequency spectrum[62;63], as do birth asphyxia and intraventricular haemorrhage[64].

As a marker of acute pain, most studies have used mean heart rate differences between groups, from a baseline period through the painful stimulus to a recovery period. Heart rate tends to increase after the stimulus, then declines again during recovery[65-68]. Heart rate responses to pain increase with increasing gestational age[65]. Heart rate variability decreases, with changes in the spectral pattern depending on the noxious event[68]. The use of analgesia attenuates characteristic heart rate responses to pain, for example using local anaesthesia during circumcision[69-71] and opioids during surgery[22;72;73]. Similar results have been obtained with non-pharmacological methods, such as soothing with the use of pacifiers and swaddling[74].

As a marker for persistent pain or distress, however, heart rate and its variability needs further study. Meanwhile, there is a role for heart rate responses as part of multi-dimensional pain assessment tools (Table 1.1).

#### *1.4.2.2 Blood pressure*

Blood pressure typically responds in parallel with heart rate to a painful event, reflecting an overall increase in sympathetic arousal[75]. As with heart rate, changes in blood pressure are not necessarily indicative of pain; for example, it may be affected by care procedures[76], medical treatment, or severe illness.

#### *1.4.2.3 Oxygen saturation*

Invasive procedures in premature infants have been shown to cause a decrease in oxygen saturation and cerebral blood flow[77].

### 1.4.3 Neurochemical measures

Faced with the challenges of using behavioural and physiological signs for assessing pain, especially chronic or persistent pain, it is no surprise that neurochemical measures have also been studied in-depth. Neurochemical measures have their own limitations. They serve mainly as a research tool, and as yet have little immediate bedside applicability. Nevertheless, if an adequately sensitive and specific neurochemical marker of pain were to be discovered, there would be further incentive to developing it for direct clinical use.

*1.4.3.1 Catecholamines*

Hypothalamic activation of the sympathetic nervous system causes an increased secretion of catecholamines from the adrenal medulla, and also the release of noradrenaline from presynaptic nerve terminals. Multiple studies have shown that neonates are indeed capable of mounting a catecholamine response to surgery[21-23;78], and that this response can be attenuated with the use of adequate analgesia[22;23], indicating that pain is almost certainly responsible. Similar responses have been found in neonates undergoing non-surgical procedures, such as mechanical ventilation[79], chest physiotherapy and endotracheal suctioning[80]. In fact, neonates have been found to exhibit a more extreme catecholamine response than adults[78].

Factors which may confound the interpretation of catecholamine levels, include hypoxia[81], and the use of dopamine[82] and pancuronium[83], which have all been shown to affect catecholamine secretion.

*1.4.3.2 Cortisol*

Cortisol is a potent glucocorticoid, accounting for 95% of endogenous glucocorticoid activity. It has been studied relatively extensively in neonates. Plasma cortisol levels in infants are affected by stress, including the stress of birth[84]. The same studies above that reported the presence of a catecholamine response to surgery in neonates also showed that elevations in plasma cortisol and other corticosteroids occurred[22;23;78]. Circumcision without anaesthesia can cause an increase in plasma cortisol[85].

*1.4.3.3 Insulin*

Insulin is a small protein with a molecular weight of 5808 Daltons, secreted by the beta cells of the pancreas. It circulates mainly in an unbound form, and has a plasma half-life of 6 minutes. Insulin secretion is affected variably in neonates following the stress of surgery, making it less reliable as a marker of pain. In association with surgery, it can be suppressed[20], unchanged[21], or elevated[23;78]. Insulin secretion is also sensitive to certain amino acids, gastrointestinal hormones, glucagon, growth hormone, cortisol, progesterone and oestrogen.

*1.4.3.4 β-endorphin and other spinal cord neuromediators*

Opioid receptors exist within the central nervous system together with multiple natural opioids. These include β-endorphin, met-enkephalin, leu-enkephalin and dynorphin. β-endorphin is an opioid peptide of 31 amino acids which derives from the same preprohormone that gives rise to adrenocorticotrophin (ACTH) and melanocyte-stimulating hormone (MSH). It also serves as a marker of the stress response in neonates[23;78].

Within the spinal cord are various other peptides and non-peptides that are involved in nociception and anti-nociception (Table 1.3). They interact in a complex manner to facilitate or inhibit the transmission of pain signals[86]. Some are detectable in plasma or saliva, such as β-endorphin, somatostatin, and CGRP. Historically though, it is SP and the other tachykinins which have been considered to hold a prominent role in the neurobiology of nociception.

**Table 1.3:** Compounds involved in nociception and anti-nociception in the spinal cord

| Peptides | Non-peptides |
|---|---|
| Opioid peptides | Norepinephrine |
| • β-endorphin | Serotonin |
| • enkephalin | α2-adrenoceptor agonists |
| • dynorphin | Acetylcholine |
| Non-opioid peptides | Nitric oxide |
| • substance P | Vanilloid receptor agonists |
| • neurokinin A | |
| • neurotensin | |
| • somatostatin | |
| • cytokines | |
| • CGRP | |
| • galanin | |
| • neuropeptide Y | |
| • cholecystokinin | |
| • purines | |
| • nociceptin | |
| Amino acids | |
| • glycine | |
| • γ-aminobutyric acid | |
| • glutamate | |
| • aspartate | |

## 1.5 TACHYKININS

### 1.5.1 Substance P (SP)

Substance P is an undecapeptide that was first described in 1931 by von Euler and Gaddum[87] as a pharmacologically active compound present in mammalian intestinal and brain extracts. Its name was derived from the working terminology used to refer to crude 'preparations' of the active material. Initially, SP was found to cause contraction of intestinal smooth muscle and lowering of blood pressure. However, the reliability of much of the research and literature available on SP before 1970 is uncertain due to the impurity of samples caused by contamination by other biologically active agents such as bradykinin.

#### *1.5.1.1 Molecular structure and classification*

A breakthrough came in 1970 when Chang and Leeman purified the peptide from bovine hypothalamus extracts and determined the amino acid sequence[88;89]. SP is classified as a member of the tachykinin family[90;91] (Table 1.4). Tachykinins were so named because they were noted to cause faster contraction of smooth muscle than bradykinin. They are peptides which have a similar amino acid sequence at the carboxyl terminus [-Phe-X-Gly-Leu-Met-$NH_2$ (X = aromatic or branched aliphatic amino acid)] and share a common spectrum of biological activity. Although most of the tachykinins initially discovered were of amphibian origin, there is now a recognised spectrum of mammalian tachykinins which include neurokinin A, neurokinin B, neuropeptide K and neuropeptide γ.

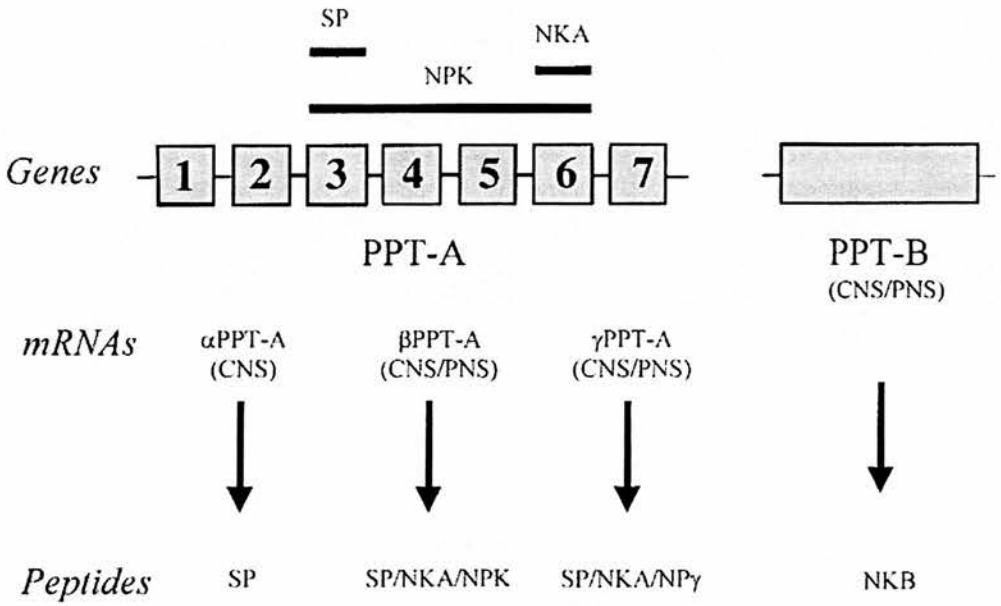
**Table 1.4:** Amino acid sequences of substance P, neurokinin A and other naturally occurring tachykinins

| | |
|---|---|
| *Mammalian* | |
| Substance P | Arg-Pro-Lys-Pro-Gln-Gln-**Phe**-Phe-**Gly-Leu-Met-$NH_2$** |
| Neurokinin A (Substance K/Neuromedin L) | His-Lys-Thr-Asp-Ser-**Phe**-Val-**Gly-Leu-Met-$NH_2$** |
| Neurokinin B (Neuromedin K) | Asp-Met-His-Asp-Phe-**Phe**-Val-**Gly-Leu-Met-$NH_2$** |
| Neuropeptide K | Asp-Ala-Asp-Ser-Ser-Ile-Glu-Lys-Gln-Val-Ala-Leu-Leu-Lys-Ala-Leu-Tyr-Gly-His-Gly-Gln-Ile-Ser-His-Lys-Arg-His-Lys-Thr-Asp-Ser-**Phe**-Val-**Gly-Leu-Met-$NH_2$** |
| Neuropeptide γ | Asp-Ala-Gly-His-Gly-Gln-Ile-Ser-His-Lys-Arg-His-Lys-Thr-Asp-Ser-**Phe**-Val-**Gly-Leu-Met-$NH_2$** |
| *Non-mammalian* | |
| Physalaemin | pGlu-Ala-Asp-Pro-Asn-Lys-**Phe**-Tyr-**Gly-Leu-Met-$NH_2$** |
| Kassinin | Asp-Val-Pro-Lys-Ser-Asp-Gln-**Phe**-Val-**Gly-Leu-Met-$NH_2$** |
| Eledoisin | pGlu-Pro-Ser-Lys-Asp-Ala-**Phe**-Ile-**Gly-Leu-Met-$NH_2$** |
| Scyliorhinin I | Ala-Lys-Phe-Asp-Lys-**Phe**-Tyr-**Gly-Leu-Met-$NH_2$** |
| Scyliorhinin II | Pro-Asp-Cys-**Phe**-Val-**Gly-Leu-Met-$NH_2$**<br>Gly<br>Asp-Pro-Cys-Lys-Ser-Asn-Ser-Pro-Ser |
| Uperolein | Glp-Pro-Asp-Pro-Asn-Ala-**Phe**-Tyr-**Gly-Leu-Met-$NH_2$** |
| Phyllomedusin | Glp-----Asn-Pro-Asn-Arg-**Phe**-Ile-**Gly-Leu-Met-$NH_2$** |

*1.5.1.2 Biosynthesis and distribution*

SP is derived from a single copy of the preprotachykinin-A (PPT-A) gene. The PPT-A gene also encodes for other tachykinins apart from SP, including neurokinin A (NKA), neuropeptide K and neuropeptide γ[92]. The preprotachykinin-B gene encodes for neurokinin B alone[93]. The PPT-A gene has seven exons that can be interchangeably spliced into one of four messenger ribonucleic acid (mRNA) transcripts: α, β, γ[94] or δ[95] (Figure 1.5). SP is encoded by exon 3, which is present in all four transcripts. This suggests that SP would be more prone to being produced whenever the PPT-A gene is expressed. Induction of the PPT-A gene is influenced by many factors, including cyclic adenosine monophosphate (cAMP), cytokines[96], thyroid hormone[97], gonadal hormones[98], glucocorticoids[99], lipopolysaccharide (LPS)[100], neuropeptides, neurotrophic factors and neuronal depolarisation. SP production in the spinal cord and increased peripheral SP release appear to be stimulated by peripheral inflammation[101;102](Table 1.5).

**Figure 1.5:** Schematic representation of the biosynthesis of SP and related peptides




From Harrison S, Geppetti P. Substance P. *International Journal of Biochemistry & Cell Biology* 2001;**33**:555-76.

**Table 1.5:** The inhibitory and excitatory effects of various biochemical compounds on peripheral SP release

| Inhibitory | Excitatory |
|---|---|
| Opiates | Heat |
| Serotonin agonists | Protons |
| Histamine agonists | High threshold mechanical stimuli |
| Dopamine agonists | Bradykinin |
| Cannabinoid agonists | Prostaglandins |
| Adrenoceptor agonists | Purines |
| K+ channel openers | Eicosanoids |
| Theophylline | Serotonin |
| Frusemide | Histamine |
| | Capsaicin |
| | Anandamide |

SP was first found to be synthesised in ribosomes of small diameter perikarya of dorsal root ganglia[103]. It is packed into storage vesicles and axoplasmically transported to peripheral and central terminations[104]. Four times as much SP accumulates peripherally compared to within the dorsal root[105]. Expression of SP and its mRNA is widespread in both the central and peripheral nervous systems. SP immunoreactivity has been detected not only in the spinal cord but also in the telencephalon, diencephalon, mesencephalon, metencephalon, myelencephalon (medulla oblongata), rhinencephalon, basal ganglia, hippocampus, amygdala,

hypothalamus and pons[106]. The areas in the central nervous system with particularly high concentrations of SP are the striatum, substantia nigra, hypothalamus and the dorsal horn of spinal cord (Figures 1.6a, 1.6b, 1.6c and 1.6d). Its production has been more recently detected in the gastrointestinal[107], immune and haematopoietic systems[108], thus identifying a possible hormonal role for it. Many subsequent studies have now shown that SP is present in various tissues in mammals.

**Figure 1.6a:** Locations of the striatum, substantia nigra and hypothalamus on a sagittal section of the brain

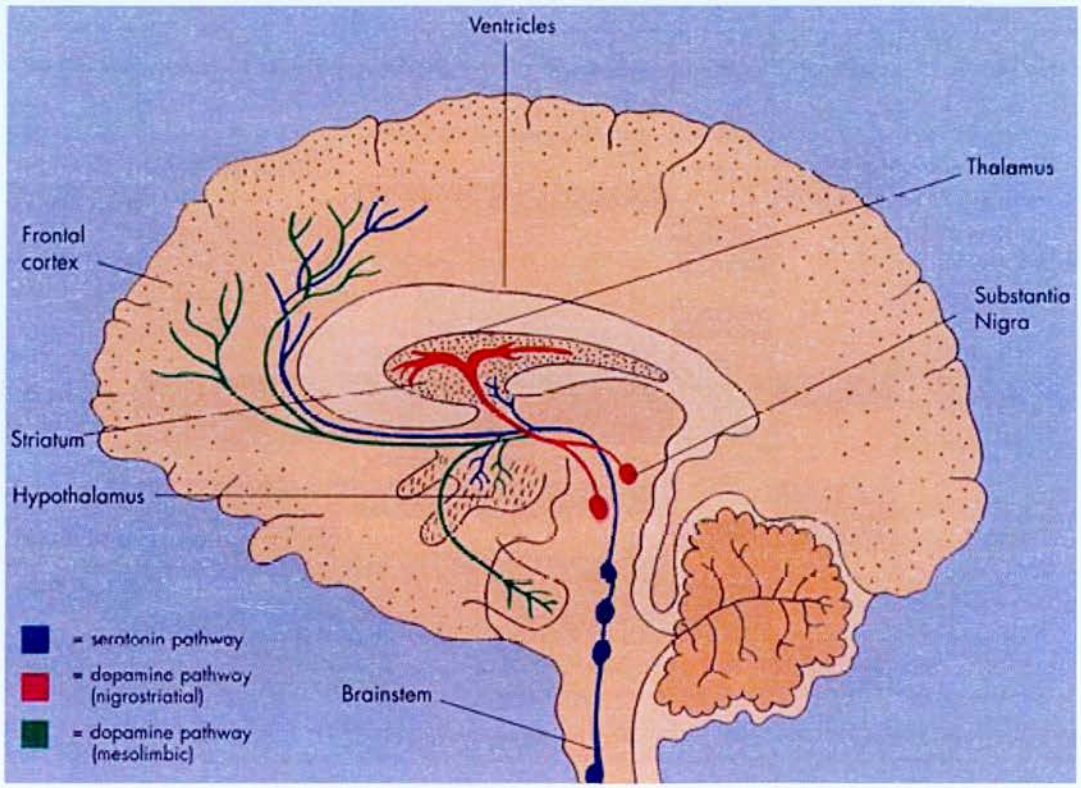


**Figure 1.6b:** Concentration of SP in the substantia nigra (in red) on a sagittal section of a rat brain

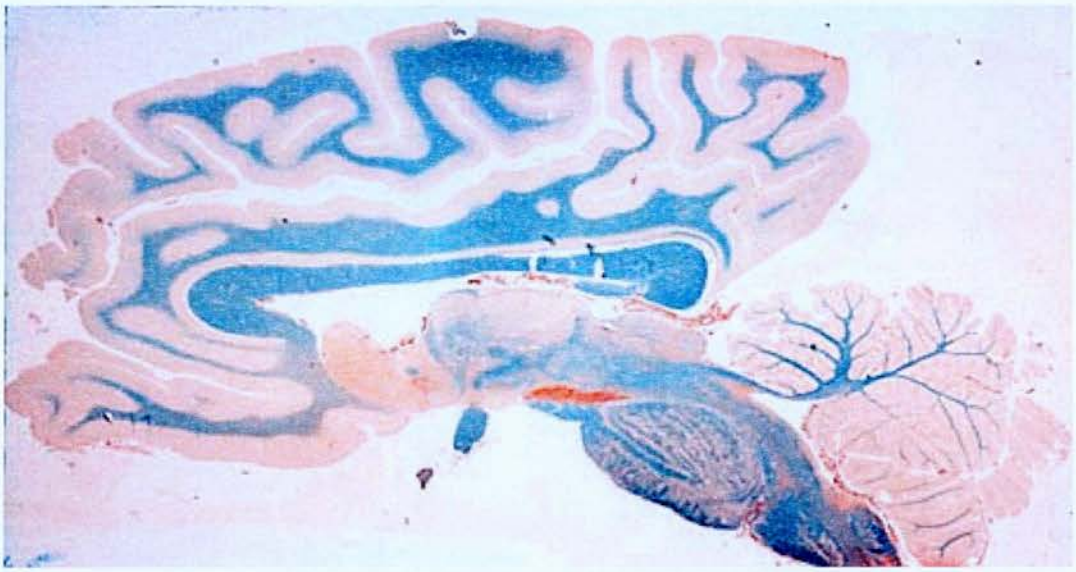


**Figure 1.6c:** Locations of the striatum, substantia nigra and hypothalamus on a coronal section of the brain (GP = globus pallidus, SN = substantia nigra, STN = subthalamic nucleus)

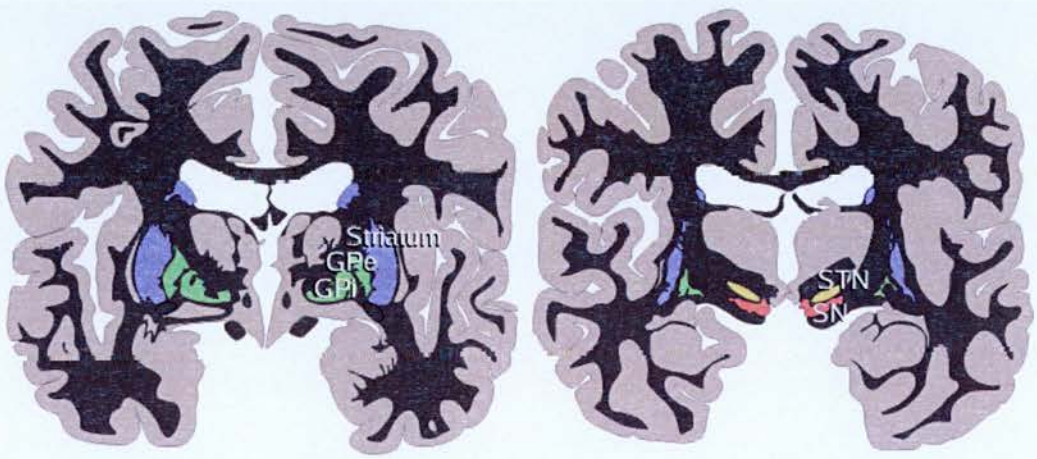
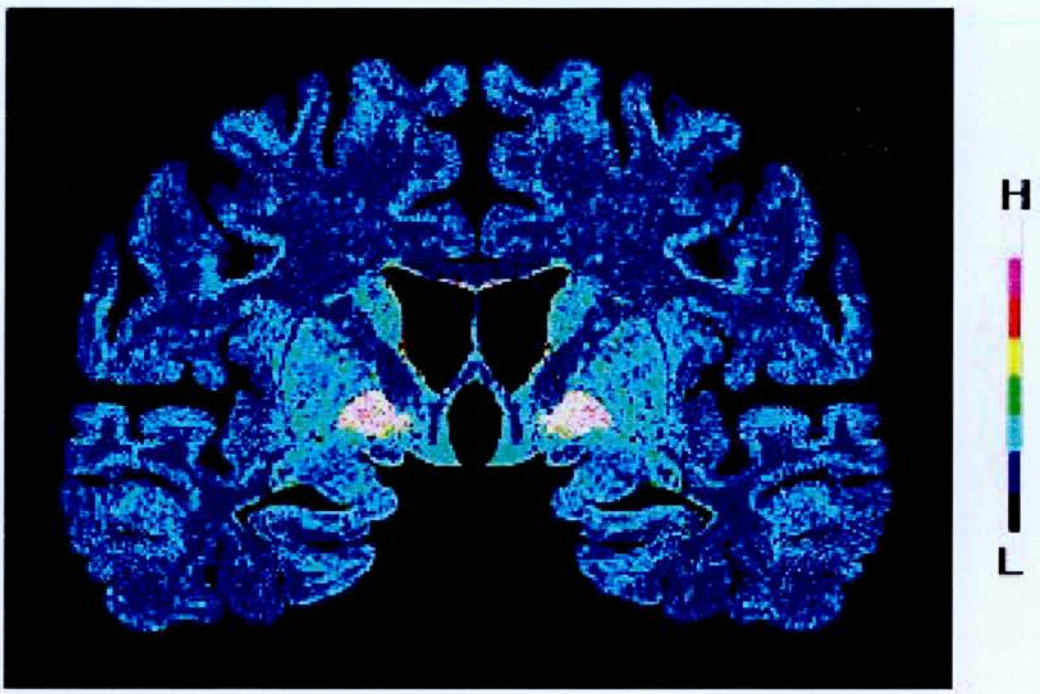


**Figure 1.6d:** Concentration of SP in the substantia nigra (in white) using the MapAnalyzer Mapping Fluorescence Microphotometry System. Quantitative immunohistochemical distribution of SP in a brain slice of an adult normal human (male, age 50). Data were obtained from approximately six million regions in the brain at 50-μm intervals. Conspicuously intense SP-like immunoreactivity was observed in the internal segment of the globus pallidus. The immunoreactive intensity in the internal segment of the globus pallidus was approximately twice as high as that in the external segment of the globus pallidus.




From Sutoo D, *et al*. Quantitative imaging of substance P in the human brain using a brain mapping analyzer. *Neuroscience Research* 1999; **35**: 339-46.

In the central nervous system, the highest concentrations of SP immunoreactivity are in the superficial dorsal horn of the spinal cord, in the substantia nigra, and in the

medial amygdaloid nucleus[109;110]. Here, SP acts fairly closely to the site of release. In the periphery, where SP occurs mainly in small diameter sensory fibres and in the gastrointestinal nervous system, it appears that SP has to diffuse through connective tissue to reach structures expressing the NK1R.

In animal studies, SP has been shown to be present together with other neuropeptides in projection neurones of visceral and taste pathways extending from the periphery to the sensory cortex in the central nervous system[111]. It appears that SP is present in neuronal cell bodies in the peripheral nodose ganglion, then the solitary nucleus of the medulla, followed by the parabrachial nucleus of the pons. However, subsequently from the thalamus projecting to the cortex, the main neuropeptides are cholecystokinin, enkephalin and somatostatin.

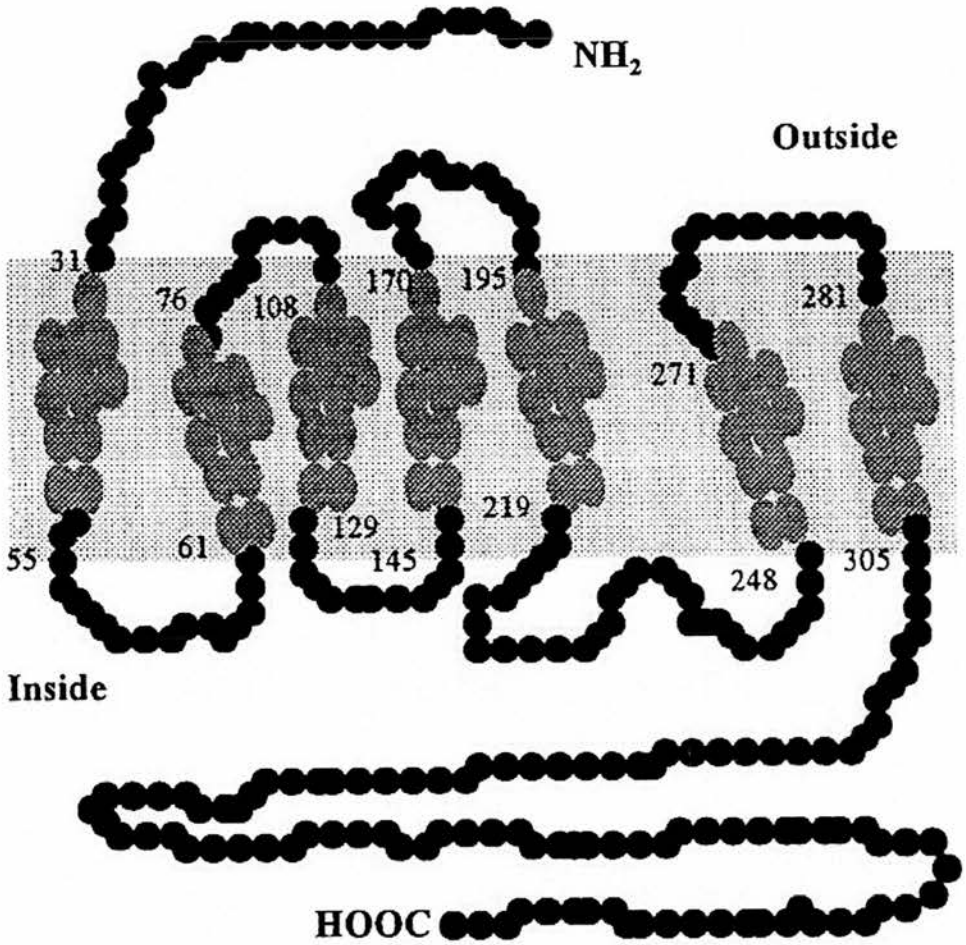
Further animal studies have shown that only approximately 20% of SP-containing sensory neurones of cervical dorsal root ganglia also possess opiate receptors and histamine receptors, indicating that nociceptors and non-nociceptive sensory inputs may be biochemically heterogeneous, hence it is unlikely that SP is confined to only having a role in nociception alone[112].

Enzymes involved in the metabolism of SP include neutral endopeptidase (NEP), SP-degrading enzyme, angiotensin-converting enzyme (ACE), dipeptidyl aminopeptidase IV, post-proline endopeptidase, cathepsin-D and cathepsin-E. The most important ones are likely to be NEP and ACE[113].

*1.5.1.3 Receptor interaction and biological activity*

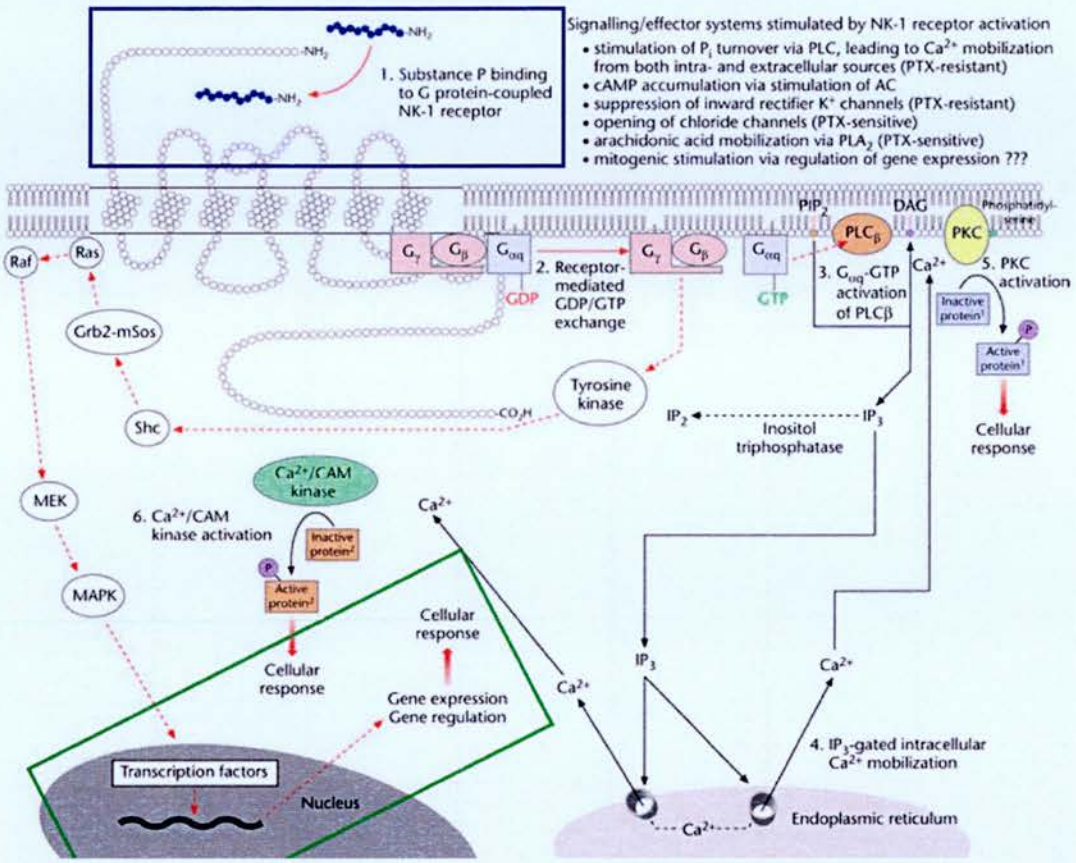
The principal receptor for SP is the neurokinin-1 receptor (NK1R), a G protein-coupled receptor with seven transmembrane domains (Figure 1.7). It is derived from a single copy gene located on human chromosome 2[114]. The neurokinin-2 and neurokinin-3 receptors (NK2R and NK3R) bind preferentially to neurokinin A and neurokinin B respectively[115] although there is some cross-reactivity. Binding of SP to NK1R evokes a signal transduction cascade and generates diacylglycerol and inositol triphosphate (Figure 1.8). These in turn activate protein kinase C and lead to release of intracellular calcium. NK1R can also mobilise cAMP and generate release of arachidonic acid. Following this, the SP-NK1R complex is internalised to endosomes where it dissociates. NK1R is recycled to the cell surface whilst SP is degraded. NK1R can undergo reversible desensitisation to SP with prolonged exposure or high concentrations.

**Figure 1.7:** Schematic diagram of the neurokinin-1 receptor




From Harrison S, Geppetti P. Substance P. *International Journal of Biochemistry & Cell Biology* 2001;**33**:555-76.

**Figure 1.8:** Signalling systems stimulated by neurokinin-1 receptor activation




From Quinn JP. The stimulus and inducible regulation of the preprotachykinin-A gene that encodes the neuropeptide substance P, and the consequences for inappropriate expression. (http://www.liv.ac.uk/neuro/members/Quinn.J.html)

High concentrations of SP-binding receptors are found in the olfactory bulb, amygdalohippocampal area and the nucleus of the solitary tract[116]. Heavy concentrations of receptors are observed in the septum, diagonal band of Broca, striatum subiculum, hypothalamus, locus coeruleus, parabrachial nucleus and lobule 9 and 10 of the cerebellum. Moderate to low concentrations of receptors are observed in the cerebral cortex, globus pallidus, raphe nuclei and the trigeminal nucleus, and

very low densities exist in most aspects of the dorsal thalamus, substantia nigra and cerebellum. Comparisons of the SP-binding receptor distribution with SP peptide levels indicate that in some areas of the brain there is a rough correlation between peptide and receptor levels. However, in other brain areas (olfactory bulb, globus pallidus and substantia nigra) there is little obvious correlation between the two.

In the spinal cord, it is now known that NKA also binds to the NK1R with equal affinity to SP and induces intracellular changes of similar magnitude[117]. Noxious stimuli in inflammatory conditions result in NKA-mediated nociception. Although SP-mediated NK1R internalisation occurs at the site of termination of stimulated nociceptors, NKA-mediated NK1R internalisation could also occur at more distant sites. NKA is also found more evenly distributed throughout the dorsal horn following a noxious insult[118].

Antagonists for NK1R were first discovered in the 1960s with an analogue of eledoisin which contained a D-amino acid[119]. However, most of the early antagonists were of limited use as they lacked oral bioavailability and central nervous system penetration (Table 1.6). The development of later antagonists such as CP96345, CP99994, RP67580 and others have opened new doors in the understanding of the physiological roles of NK1R. The NK1R has been shown to be involved in pain[120], hyperalgesia[121], depression[122] and anxiety states[123], colitis[124], emesis[125] and arthritis[126]. From human studies, NK1R antagonists have been found to be more effective in persistent pain states rather than acute pain, again reinforcing the involvement of SP in persistent pain[127].

**Table 1.6:** NK1 and NK2 receptor antagonists

| NK1R antagonists | NK2R antagonists |
|---|---|
| Aprepitant | SR48968 |
| L759274 | MEN11420 |
| RP67580 | MEN10376 |
| L733138 | MEN10627 |
| SDZNKT34311 | GR98400 |
| CGP49823 | L659874 |
| MK0869 | |
| LY303870 | |
| SR140333 | |
| TAK637 | |
| CP99994 | |
| L733060 | |
| L703606 | |
| CP96345 | |
| GR205171 | |
| PD154075 | |
| GR82334 | |
| GR203040 | |
| WIN51708 | |
| WIN62577 | |
| L668169 | |

| |
|---|
| FK888 |
| FR113680 |
| GR71251 |
| Spantide |

The main peripheral effect of SP is to produce neurogenic inflammation, resulting in vasodilation, plasma extravasation, leucocyte adhesion, smooth muscle contraction and glandular secretion. However, micromolar concentrations of SP are required to evoke plasma extravasation, whilst dermal C-fibres release only nanomolar concentrations.

*1.5.1.4 Role in nociception*

Initial theories about SP as a 'pain transmitter' arose in the 1950s because of its concentration in the dorsal rather than ventral roots of the spinal cord[128]. Since then, numerous animal studies have shown that SP is released with NKA *in vivo* in response to noxious peripheral stimulation. There is electrophysiological evidence to show that SP can activate dorsal horn neurons[129;130]. Also, morphine and related analgesics were found to inhibit SP release from sensory terminals *in vitro*[131]. Cells which respond most sensitively to SP are also the ones which respond most strongly to painful stimuli[132]. From NK1R and NK2R antagonist studies, SP and NKA have been implicated in the spinal nociceptive reflex[133;134]. SP and NKA are preferentially involved in mediating moderate to intense pain, as opposed to mild pain, and they play an important role in persistent pain caused by tissue injury[130].

Studies in human subjects indicate that SP has more to do with chronic pain associated with inflammation than with acute pain such as angina[135], labour pain[136] and postoperative pain[136]. The majority of studies into SP and pain have been animal studies, and what has been investigated in humans has given rise to conflicting results. This is probably due to the existence of some cross-species variation in tachykinin distribution and hence results from animal studies cannot necessarily be extrapolated to humans. Further difficulties giving rise to conflicting results may be due to sampling methods, and sample processing and analysis. The tachykinins are rapidly degraded in plasma and saliva, hence adequate sample preservation is required. Also, various methods of extracting peptide (with various degrees of peptide recovery), and of assaying peptide, have been reported.

Thus with regard to chronic pain in human subjects, some researchers have shown that SP decreases, e.g. plasma SP in chronic back pain[137], and others that it increases, e.g. plasma SP in chronic arthritic pain[138], cerebrospinal fluid (CSF) SP in various painful spinal diseases[139], and CSF SP in fibromyalgia[140]. Moreover, a study by Nicolodi and Del Bianco[141] showed that salivary SP in migraine and cluster headache sufferers was at a level similar to controls during attacks, but at a lower basal level in between attacks. SP may also have a role in increasing pain threshold and tolerance locally[142]. In an interesting case report of congenital insensitivity to pain, there was absence of NK1R in the skin of the patient[143].

More recently, Basbaum[144] has proposed that persistent injury causes reorganisation in the spinal cord circuitry which results in exacerbated pain, and that SP plays a part

in this. SP is released by intense stimulation of somatic and visceral tissues by various modalities, which would certainly manifest as severe pain. Inflammation then contributes by increasing dramatically the numbers of nerve cells involved. Furthermore, activation of NK1R at the spinal level contributes to the induction and expression of opioid physical dependence[145].

*1.5.1.5 Gastrointestinal effects*

SP was first found in the gastrointestinal system[87]. SP is expressed in distinct neural pathways in all the layers of mammalian gut as well as in the extrinsic primary afferent nerve fibres innervating the gut vascular system[146]. Here, SP is likely to co-mediate gut motility, secretory processes and inflammation together with other enteric transmitters[147]. SP may also be involved in inflammatory bowel disease with findings of increased SP in left-sided ulcerative colitis and increased expression of NK1R in the gut vasculature and lymphoid system in ulcerative colitis and Crohn's disease. NK1R is also upregulated in *Clostridium difficile*-induced pseudomembranous colitis[148].

*1.5.1.6 Influence on haematopoiesis, the immune system, and the inflammatory response*

The mechanisms by which SP links the neural-immune-haematopoietic axis are summarised in reviews by Payan[149] and Rameshwar[108]. SP is released by nerve fibres in lymphoid organs and also produced by immune cells[100;150]. It is closely involved in communication with other molecules such as cytokines and adhesion factors. This network is the regulator of the inflammatory response, resulting in recruitment of

polymorphonuclear leucocytes and subsequent infiltration by macrophages and T-cells. Delineated functions of SP in the immune system include:

a) induction of cytokine production (which can in turn further induce NK1R production),

b) stimulation of lymphocyte production,

c) enhancement of chemotaxis and phagocytosis,

d) induction of immunoglobulin production through B-cell interaction,

e) promotion of eosinophil migration, mast cell degranulation and histamine release, and

f) regulation of tissue repair via increased fibroblast, smooth muscle cell and endothelial cell proliferation.

It is probably through these processes that SP has its role in inflammatory conditions such as arthritis, asthma and inflammatory bowel disease.

In a similar manner, SP regulates haematopoiesis in conjunction with other cytokines. It can be found in marrow stroma as well as nerve fibres. Here, SP exerts a positive influence on bone marrow in that it stimulates production of progenitor cells.

*1.5.1.7 Effects on the central nervous system*

The effects of SP on memory, reinforcement and brain dopamine activity are outlined in a review by Huston *et al*[151]. SP appears to have a memory-promoting effect in parallel with increased site-specific extracellular dopamine. This might account for the impairment in associative functioning in some neurodegenerative diseases. SP is also known to be involved in depression, nausea and vomiting, and

new therapies for these problems are being developed in the form of NK1R antagonists[152;153]. Aprepitant (Emend, Merck & Co., Inc.), the first of this class, was recently approved by the United States Food and Drug Administration (FDA) for the prevention of both acute and delayed chemotherapy-induced nausea and vomiting[154]. More recently, the antagonist L759274 has been shown to be efficacious as an anti-depressant in a randomised, double-blinded placebo-controlled trial[155].

*1.5.1.8 The respiratory system and the Sudden Infant Death Syndrome*

SP has been implicated in pulmonary inflammation[156], asthma[157] and the sudden infant death syndrome (SIDS)[158;159]. SP release in the airways can be induced by noxious factors such as viruses and chemicals. The importance of SP in pulmonary neurogenic inflammation was defined in an animal study by Bozic *et al*[160], in which normal mice were compared with NK1R-knockout mice. The inflammatory response stimulated by immune complexes in normal mice was absent in NK1R-knockout mice, suggesting that SP is vital in the amplification of pulmonary inflammation induced by immune complexes. This also implies that blocking the SP system could be useful in preventing adverse effects of pulmonary inflammation, but then the protective side of the inflammatory response might be compromised. Subsequent studies using NK1R antagonists have shown that airway hyper-reactivity and microvascular permeability can be abolished[161;162].

Through its effect on smooth muscle contraction, SP has been found to cause constriction of human bronchi *in vitro*[163] and guinea pig airways *in vivo*[164]. It has been proposed that this is part of the axon reflex mechanism thought to be

contributory to the pathophysiology of asthma. Damage to airway epithelium would allow C-fibre afferent nerve endings to be exposed to irritant stimuli and hence produce SP and other sensory neuropeptides, which in turn produce bronchoconstriction[157]. SP also increases the tracheal secretion of epithelial and glycoprotein-rich fluids, and induces the release of mediators such as prostaglandins and nitric oxide from the airway epithelium[157]. High concentrations of SP have resulted in inhibition of surfactant secretion from isolated type II pneumocytes in animal models[165].

Although the principal neurotransmitters involved in producing the respiratory cycle are glutamate, gamma-aminobutyric acid (GABA) and glycine, SP has been found to be a modulator by acting both centrally and on peripheral sensory receptors[166]. In the central nervous system, SP enhances the respiratory cycle by increasing the respiratory rate as well as tidal volume. SP release from carotid chemoreceptor nerves also occurs in response to hypoxia. It has been postulated that SP may be involved in the pathophysiology of SIDS and there have also been studies performed into SP and brainstem gliosis in SIDS[158;159].

*1.5.1.9 Cardiovascular effects*

Species variation exists in the expression of SP in the mammalian heart. SP appears to have no direct effect on heart muscle, but is a potent vasodilator. The vasodilatory responses are brought about via NK1R in large arteries, leading to nitric oxide release from the endothelium[167].

*1.5.1.10 Relationships with other hormones*

There is a huge amount of literature about SP and its interactions with other hormones. Its role in the hypothalamic-pituitary axis has been reviewed by Jessop *et al*[168]. SP modulates luteinising hormone (LH) and follicle-stimulating hormone (FSH) release and is in turn modulated by oestrogen. Similarly, SP promotes thyrotrophin (TSH) release, and pituitary SP content is decreased by thyroxine and increased by thyroidectomy. SP appears to centrally mediate the inhibition of ACTH release from the anterior pituitary, probably by inhibiting corticotrophin-releasing hormone production in the hypothalamus. Again, anterior pituitary SP is decreased by adrenalectomy. SP also either stimulates or inhibits release of prolactin and growth hormone in what appears to be a dose-dependent relationship.

## 1.5.2 Neurokinin A (NKA)

*1.5.2.1 Molecular structure and receptor interaction*

Spliced from the same gene that codes for SP (PPT-A gene), NKA is a decapeptide and a tachykinin very closely related to SP (Table 1.4). It is also known as substance K or neuromedin L. The term neurokinin was first used in 1960 to describe 'a polypeptide formed during neuronal activity in man' but that this was one of the tachykinins is unclear[169]. Purified from porcine spinal cord, the amino acid sequences of NKA and NKB were formally determined in 1983 by separate research groups, and they were shown to have 81% and 65% of the activity of SP respectively on guinea pig ileum[170-172]. As early as June the same year, it was postulated that NKA

acted on a different receptor to SP[173;174]. The separate NK2 receptor for NKA was identified in 1987[175].

*1.5.2.2 Biological activity*

NKA is generally co-synthesised, co-localised, and co-secreted with SP. It has similar effects to SP in that it also causes smooth muscle contraction, hypotension, bronchoconstriction and plasma extravasation[176]. However the variation in regional distribution of SP, NKA and NKB in rat brain and spinal cord suggested that each tachykinin has a specific physiological function[177]. NKA has a neurotransmitter role in the spinal cord, seen both by a depolarising response measured from lumbar ventral roots following NKA application[178], and also on immunohistochemistry[118;179]. Evidence that both SP and NKA were involved in the transmission of pain signals in C-fibres in the spinal cord accumulated[118;180;181].

It was therefore surprising to find that NK2R exists minimally in the spinal cord[182]. There is, however, some cross-reactivity between the tachykinins and the NK receptors. It was subsequently confirmed that NKA is a high-affinity ligand for the NK1R but that it is a poor competitor for SP[183]. NKA does contribute to at least 50% of the NK1R activation in the dorsal horn of the spinal cord and is an important NK1R ligand *in vivo*[117;184]. Furthermore, it has been demonstrated that SP and NKA may have a synergistic effect on spinal cord excitability[185]. As detailed in Section 1.5.1.3, it seems plausible that it is NKA, rather than SP, which is responsible for nociceptive information transmission in the spinal cord.

### 1.5.3 Substance P and neurokinin A in infants

If little work has been done on SP and NKA in adults, even less has been studied in neonates and infants. There are no *in vivo* studies on NKA in infants or children. Most of the SP studies have been confined to *in vitro* experiments such as anatomical mapping of SP dense areas in various parts of the body and post-mortem investigations in SIDS as outlined above.

The most recent publication related to *in vivo* studies in children was on age-dependent levels of neuropeptides (including SP) in normal children. The authors attempted to describe normal values using cord blood from six full-term infants, and peripheral blood from 41 children varying in age from 1 month to 21 years[186]. An early study on CSF SP levels found that levels were 100 to 1000-fold higher in fetuses and preterm infants than in adults[187]. A later study on CSF SP levels in 39 neurologically normal children showed that levels of SP and β-endorphin peak in the first year of life and subsequently decline[188]. Finally, plasma and CSF SP levels were shown to be elevated in 70 children with seizure disorders[189]. Again, this study was difficult to interpret as it had subjects ranging in age from 1 month to 18 years.

Focusing on infants, only one very brief paper into plasma SP levels in healthy term and preterm babies has been produced[190], but unfortunately the data are unhelpful for the following reasons:

a) the gestation of the preterm group was reported to be < 37 weeks with no lower limit defined nor subgroups considered,

b) the investigations were performed any time after the third week of life and results were plotted according to the infants' corrected gestational age (which ranged from –4 to 63 weeks (0 being term)),

c) although the two groups were described as healthy babies, there were no qualifications to this, which would be especially relevant in the preterm group, and

d) there was very little description of the methods of sample collection, preservation and analysis used and degree of consistency in this.

## 1.6 AIMS AND OBJECTIVES OF THE CURRENT STUDY

The aims of this research project were:

a) to develop radioimmunoassays for SP and NKA sufficiently sensitive to detect the neuropeptides from neonatal microsamples of plasma and saliva,

b) to determine variations in SP and NKA with gestational and postnatal age,

c) to study whether plasma and saliva SP and NKA levels varied with persistent pain in neonates, and could therefore serve as potential markers of pain.

Currently, no gold standard exists in the measurement of chronic or persistent pain in newborn infants. It is therefore difficult, if not impossible, to establish which infants are suffering persistent pain sufficient to be of physiological or psychological importance. Yet it is important to try to develop a measure that could be reliable enough to guide a clinician to treat chronic pain where warranted. The PIPP is the only measure which has been validated for use in more persistent pain, i.e. post-

operative pain, and is therefore the closest to a gold standard measure that is available.

# 2. DEVELOPMENT OF PEPTIDE EXTRACTION PROCEDURE

Peptide extraction is a prerequisite for most radioimmunoassays. Extraction serves to purify the sample and to ensure that the matrix of the sample is as similar to the assay standards as possible. This is particularly important if the antibody is not highly specific. Sample extraction can be performed using various methods, e.g. liquid-liquid phase extraction, solid phase extraction columns with various liquid mobile phases, or high-performance liquid chromatography.

The main disadvantages of sample extraction include loss of sample, and it is often a time-consuming process. Loss of sample can be minimised by fastidious determination of the best procedure to extract a particular peptide. This involves investigating different physical methods of peptide extraction, as well as different variants within a method.

Materials used are detailed in Appendix A.

## 2.1 DETERMINING THE NEED FOR AN EXTRACTION PROCEDURE

This is performed by serially diluting the raw samples to be analysed, e.g. plasma or saliva, and measuring peptide concentrations within an assay. If the antibody is specific enough for the peptide in question, the serial dilution of samples will yield a corresponding serial reduction in measured peptide. If the antibody is cross-reacting

significantly with a matrix component, there will not be a serial reduction in measured peptide. For choice of antibody, see Section 3.1.

Pooled plasma and saliva samples taken from adult volunteers were spiked with a known concentration of SP standard and serially diluted 1:1 with assay buffer. Each dilution was assayed in quintuplicate. Measured SP concentrations are tabulated below (Table 2.1).

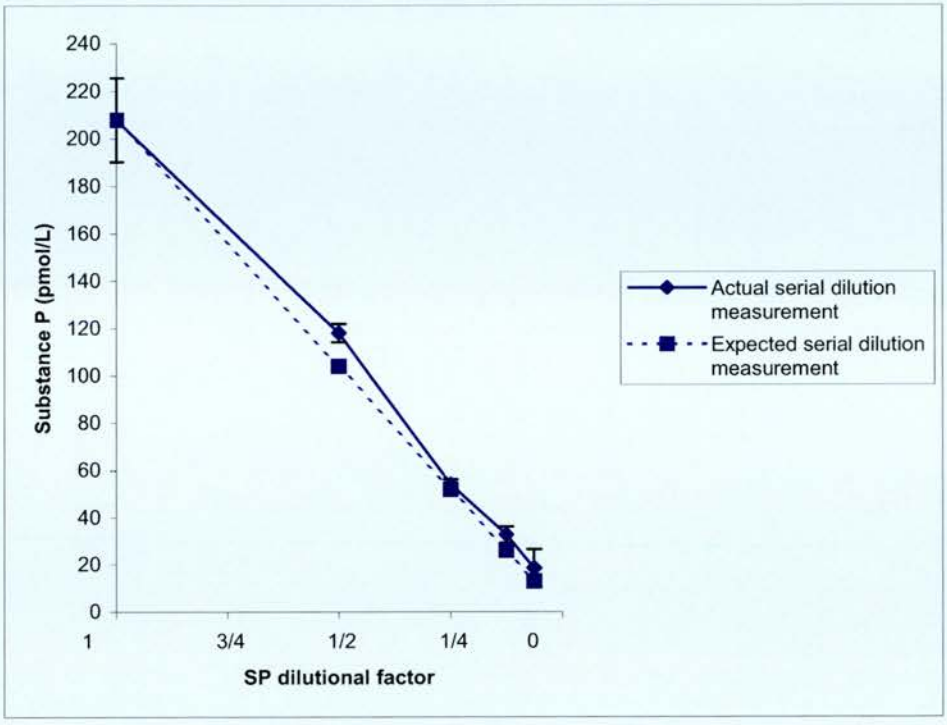
**Table 2.1:** SP concentrations in serially diluted plasma and saliva samples measured by direct assay

| Matrix | SP dilutional factor | Measured SP concentration (pmol/L) Mean ± 1SD |
|---|---|---|
| Plasma | 1 | 207.9 ± 17.6 |
| | 1:2 | 118.0 ± 3.9 |
| | 1:4 | 54.0 ± 2.0 |
| | 1:8 | 32.8 ± 3.4 |
| | 1:16 | 18.6 ± 8.0 |
| Saliva | 1 | 153.5 ± 6.7 |
| | 1:2 | 85.8 ± 9.3 |
| | 1:4 | 56.2 ± 2.6 |
| | 1:8 | 39.8 ± 3.5 |
| | 1:16 | 23.8 ± 3.3 |

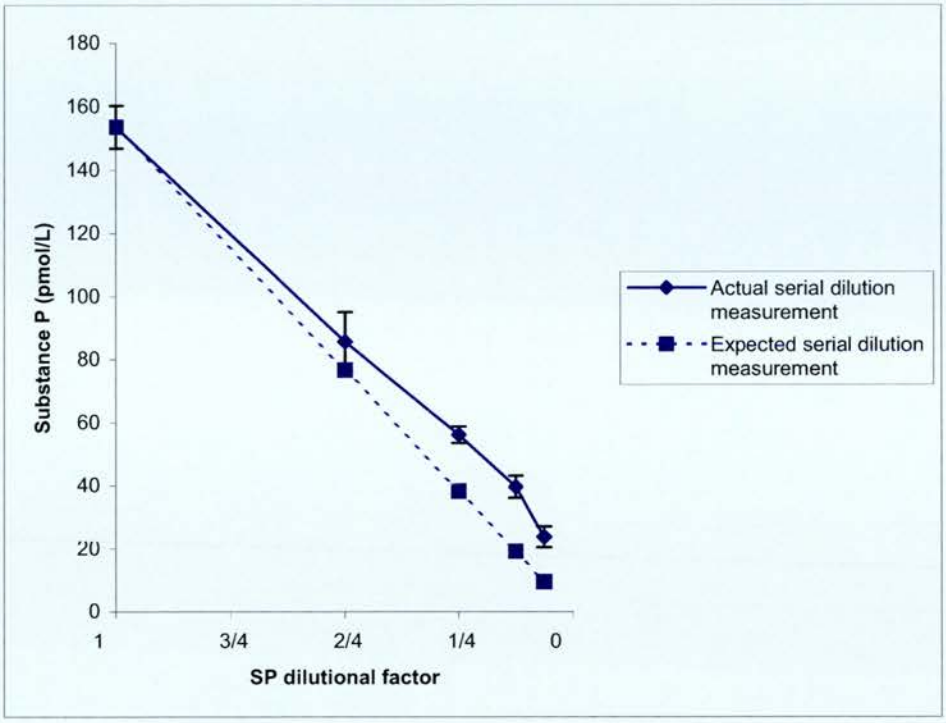
SPDA2

When these results are displayed graphically, it is seen that serial dilutions of plasma and saliva samples spiked with SP do not yield a serial reduction in measured peptide when assayed directly (Graphs 2.1 and 2.2). This indicates that the antibody does cross-react with a matrix component and hence sample extraction is warranted. This is further confirmed in Section 2.2.1.

**Graph 2.1:** Direct assay of whole plasma spiked with SP and serially diluted



**Graph 2.2:** Direct assay of whole saliva spiked with SP and serially diluted




## 2.2 LIQUID PHASE EXTRACTION (LPE) PROCEDURE

Parris *et al*[137] described a liquid phase extraction procedure developed by Yanaihara *et al*[191] and recommended by the commercially produced SP radioimmunoassay kit produced by Incstar Corp., Stillwater, Minnesota, USA. This extraction method reportedly yielded a high percentage peptide recovery (Appendix B).

### 2.2.1 Specificity of peptide recovery using serial dilution

As in Section 2.1, serial dilution of pooled plasma and saliva samples spiked with SP standard was performed. Half of each dilution underwent LPE. The other half was

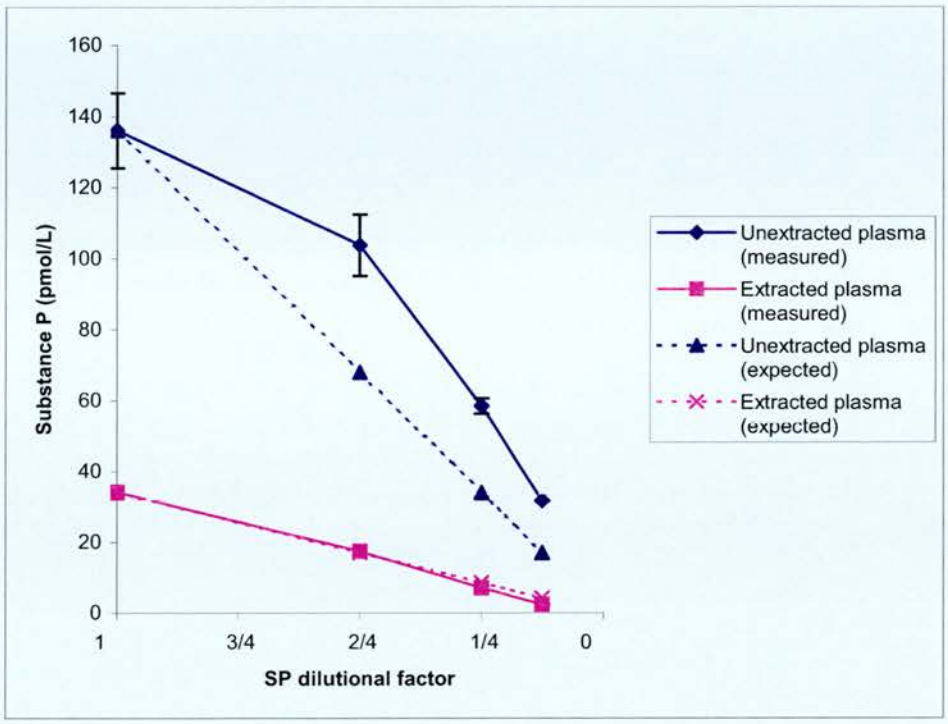
assayed directly, i.e. unextracted, and the corresponding results displayed. The results are shown in Table 2.2, and Graphs 2.3 and 2.4.

**Table 2.2:** SP concentrations in serially diluted plasma samples measured by radioimmunoassay following liquid phase extraction versus no extraction

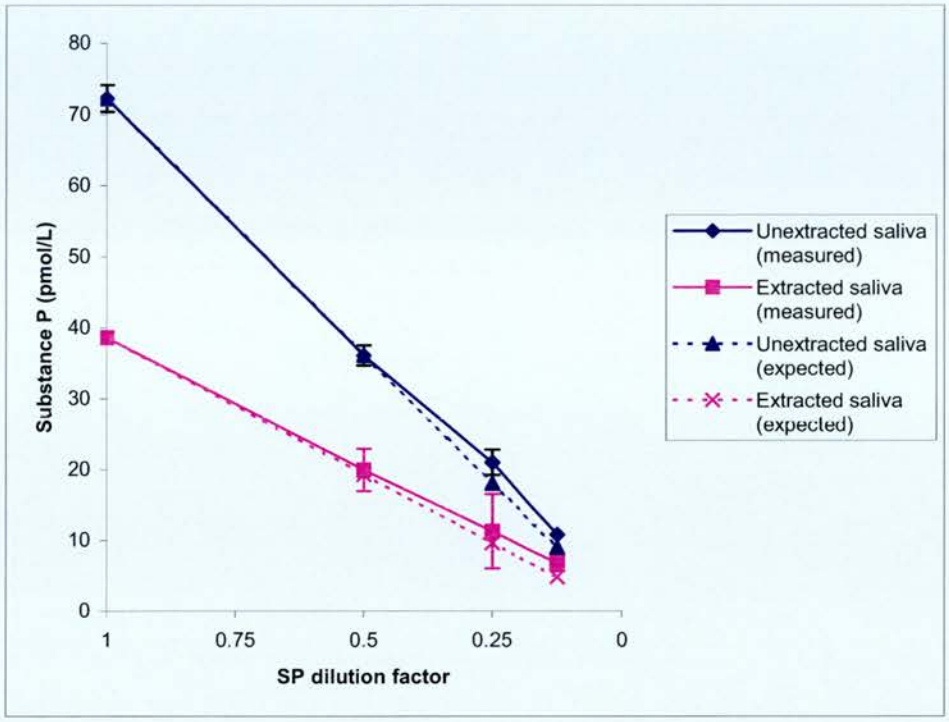
| **Matrix** | **SP dilutional factor** | **Measured SP concentration (pmol/L) Mean ± 1SD** | |
|---|---|---|---|
| | | **Extracted samples** | **Unextracted samples** |
| Plasma | 1 | 34.216 ± 0.65 | 136.015 ± 10.5 |
| | 1:2 | 17.481 ± 0.68 | 103.75 ± 8.66 |
| | 1:4 | 7.108 ± 0.63 | 58.467 ± 2.15 |
| | 1:8 | 2.426 ± 0.21 | 31.727 ± 0.13 |
| Saliva | 1 | 38.599 ± 0.74 | 72.255 ± 1.9 |
| | 1:2 | 19.952 ± 2.99 | 36.081 ± 1.43 |
| | 1:4 | 11.266 ± 5.25 | 21.017 ± 1.78 |
| | 1:8 | 6.789 ± 1.16 | 10.813 ± 0.0 |

SPLEFA16

**Graph 2.3:** SP concentrations in serially diluted plasma samples measured by radioimmunoassay following liquid phase extraction versus no extraction



**Graph 2.4:** SP concentrations in serially diluted saliva samples measured by radioimmunoassay following liquid phase extraction versus no extraction




SP recovery for the same samples were as follows:

**Table 2.3:** SP recovery

| Sample type | Recovery of SP (%) |
|---|---|
| Extracted plasma | 77.9 |
| Extracted saliva | 85.2 |
| Unextracted plasma | 170.3 |
| Unextracted saliva | 87.9 |

These results confirmed that sample extraction was essential for plasma samples as:

i) 'recovery' of SP was in excess of 100% (indicating antibody cross-reactivity with a matrix component)

ii) serial dilution of plasma samples did not yield a serial reduction in measured SP concentration when not extracted, and

iii) the measured SP concentration of plasma was four times higher when not extracted compared with extracted samples.

For saliva samples, it may appear at first glance that extraction is not vital, as serial reduction in measured SP concentrations is achieved. However, as with plasma, the unextracted samples yielded almost double the measured SP concentrations of extracted samples, indicating significant matrix cross-reaction once again.

Extraction for both plasma and saliva samples was therefore determined to be a pre-requisite for subsequent SP and NKA radioimmunoassay.

### 2.2.2 Determination of percentage recovery using 'hot' plasma and saliva

The degree of peptide recovery following liquid phase extraction was first assessed crudely using 'hot' plasma and saliva, i.e. spiked with equal volumes of $^{125}$I-SP to approximately 6000 cpm. The samples were extracted using the method described above (Appendix B), and extracts and protein precipitates counted separately at the end of the procedure to identify where peptide losses occurred, if any. Table 2.4 shows percentage recovery in the extracts at the end of the liquid phase procedure.

**Table 2.4:** Percentage recovery from liquid phase extraction procedure (Yanaihara) using 'hot' plasma and saliva samples

| Matrix | Matrix volume (µl) | Extract recovery (%) | Mean extract recovery ± 1SD (%) |
|---|---|---|---|
| **Plasma** | 500 | 79.66 | |
| | 500 | 82.66 | |
| | 500 | 80.19 | |
| | 500 | 81.09 | |
| | 500 | 82.09 | 81.45 ± 1.24 |
| | 250 | 81.79 | |
| | 250 | 81.10 | |
| | 250 | 80.81 | |
| | 250 | 81.15 | |
| | 250 | 83.95 | |
| **Saliva** | 500 | 100.34 | |
| | 500 | 96.68 | |
| | 500 | 98.57 | |
| | 500 | 98.55 | |
| | 500 | 100.78 | 96.81 ± 4.06 |
| | 250 | 97.28 | |
| | 250 | 97.48 | |
| | 250 | 96.73 | |
| | 250 | 86.26 | |
| | 250 | 95.38 | |

SPLE2&3

The liquid phase extraction procedure therefore looked promising, with relatively good recovery yields, though not as high as claimed in the Yanaihara publication[191]. There was a marked difference in recovery between plasma and saliva samples. The residual counts that were lost were in the protein precipitate formed in the first step of the extraction. Plasma has more protein than saliva and hence yielded larger precipitates. The mean percentage of total counts present in the protein precipitates were 17.46% for plasma and 5.07% for saliva. Using this liquid phase extraction procedure, significant amounts of peptide were therefore not being extracted, but lost in protein precipitation.

### 2.2.3 Determination of percentage recovery using 'cold' plasma and saliva

The experiment was subsequently performed using plasma and saliva spiked with 100 pmol/L of SP standard instead of $^{125}$I-SP (i.e. 'cold' plasma). The samples then underwent liquid phase extraction and SP concentrations were measured by radioimmunoassay. Table 2.5 shows percentage recovery in the extracts. The original SP concentrations in the plasma and saliva samples without spike added were represented by $x$ and $y$ pmol/L respectively. In the spiked samples, the volume of spike used was 20% of the total pre-extraction sample volume. Hence the original SP concentration in the spiked samples is represented by $0.8x+100$ and $0.8y+100$ pmol/L respectively for plasma and saliva.

**Table 2.5:** Percentage recovery from liquid phase extraction procedure (Yanaihara) using 'cold' plasma and saliva samples measured by RIA

| Matrix | Original SP concentration (pmol/L) | Measured SP concentration (pmol/L) | Mean SP concentration (pmol/L) |
|---|---|---|---|
| **Plasma** | x | 8.952 | |
| | x | 7.590 | |
| | x | 16.408 | 10.41 |
| | x | 8.323 | |
| | x | 10.786 | |
| **Saliva** | y | 27.200 | |
| | y | 20.238 | |
| | y | 19.081 | 20.36 |
| | y | 14.531 | |
| | y | 20.765 | |

| | | | Extract recovery (%) | Mean extract recovery ± 1SD (%) |
|---|---|---|---|---|
| **Spiked plasma** | 0.8x+100 | 96.318 | 87.99 | |
| | 0.8x+100 | 100.440 | 92.11 | |
| | 0.8x+100 | 98.393 | 90.06 | 87.95 ± 5.9 |
| | 0.8x+100 | 86.108 | 77.78 | |
| | 0.8x+100 | 100.160 | 91.83 | |
| **Spiked saliva** | 0.8y+100 | 88.571 | 72.28 | |
| | 0.8y+100 | 94.448 | 78.16 | |
| | 0.8y+100 | 81.903 | 65.61 | 68.14 ± 7.3 |
| | 0.8y+100 | 75.460 | 59.17 | |
| | 0.8y+100 | 81.768 | 65.48 | |

SPLEFA10

The recovery in plasma samples was comparable to that achieved with 'hot' plasma samples. However, the recovery with saliva was much poorer than with 'hot' saliva samples.

## 2.3 SOLID PHASE EXTRACTION (SPE) PROCEDURE

Having achieved unsatisfactory peptide recovery with liquid phase extraction, the efficiency of solid phase extraction was investigated. In solid phase extraction, the sample is passed through a column (the solid phase), during which time peptides bind with the column matrix via ionic interactions. The column is then washed of residual plasma. Subsequently, the peptides bound to the column matrix are released, or eluted, by a mobile phase (eluant) which reverses the ionic interactions. The resultant eluate is collected, dried, and subsequently assayed for the required peptide.

As such, various solid phase columns exist, differing in the matrix component depending on the type of peptide or other molecule to be extracted. An ideal column has a high affinity for the peptide of interest, and the corresponding ideal eluant must be able to elute the peptide from the column to completion. In addition, the wash used in between must not be able to elute the peptide from the column.

Various SPE column manufacturers were contacted to obtain information on which types of column would be most suitable for the extraction of tachykinins. Samples were provided with which to run preliminary extractions to determine column suitability.

### 2.3.1 Comparison between C8 and C18 SPE columns

In the first instance, the columns investigated were C8 and C18 types as they were most suitable for small peptides. Two ml of assay buffer spiked with approximately 2250 cpm of $^{125}$I-SP was run through each column type in duplicate, following the

extraction procedure recommended by Euro-Diagnostica (Appendix C). Loss of peptide during the extraction procedure was determined by measuring gamma counts in the post-extraction samples and the column matrix, as well as the eluate. The results are tabulated below.

**Table 2.6:** Source of peptide losses during solid phase extraction with C8 versus C18 columns

| **Column type** | **% of pre-extraction counts** | | | **Total %** |
|---|---|---|---|---|
| | Post-extraction plasma | Retained on column | Eluate recovery | |
| C18 MF 200 mg | 5.2 | 110.4 | 4.9 | 120.5 |
| C18 MF 200 mg | 5.4 | 111.6 | 4.3 | 121.3 |
| C8 EC 100 mg | 23.1 | 8.6 | 74.9 | 106.6 |
| C8 EC 100 mg | 61.6 | 5.6 | 44.8 | 112.1 |

SPSE4

From this, it could be seen that the residual post-extraction sample contained more SP after passing through C-8 columns, indicating that these columns had a lower affinity for SP and hence were unable adequately to extract the peptide from the sample in the first stage. Conversely, C-18 columns appeared to have a high extraction ability for SP, leaving little in the post-extraction sample. However, it became clear from this experiment that although the C-18 columns extracted the peptide, the eluant used was ineffective in the last stage of the extraction procedure. A different eluant was required.

### 2.3.2 Comparison between different C18 SPE columns

Five different C18 SPE columns were tested for SP recovery using 'hot' plasma spiked with equal volumes of $^{125}$I-SP to approximately 8000 cpm. The mobile phase according to Fehder was used (Appendix D). Table 2.7 shows percentage recovery from various components of the procedure.

**Table 2.7:** Percentage recovery of $^{125}$I-SP from various components of C18 SPE procedure

| **Manufacturer** | **SPE column** | **Eluate recovery (%)** | **Post-extraction plasma (%)** | **Retained on column (%)** | **Total %** |
|---|---|---|---|---|---|
| Isolute | C18 NEC 50 mg | 75.1 | 20.9 | 3.1 | 99.1 |
| Isolute | C18 MF 200 mg | 64.6 | 10.2 | 11.0 | 85.8 |
| Varian Bond-elute | C18 OH 100 mg | 89.1 | 11.4 | | 100.5 |
| Phenomenex Strata | C18 M 50 mg | 88.8 | 13.8 | | 102.6 |
| Isolute | PRS 50 mg | 7.4 | 25.3 | 60.0 | 92.7 |

SPSECC

The Isolute C18 NEC, Varian Bond-elute C18 OH, and Phenomenex Strata C18 M yielded the highest recoveries with 'hot' plasma. The Isolute PRS was not evaluated further as it yielded a very poor eluate recovery, with corresponding high losses of SP in the post-extraction sample. This was replaced with the Varian C18 50 mg in the next stage.

Five C18 SPE columns were then tested for SP recovery using 'cold' plasma, spiked with 50 pmol/L of SP standard. Another five columns were simultaneously tested using 'cold' saliva. To assess recovery, plasma and saliva from the same pool, but without any addition of standard, were also extracted to obtain concentrations of endogenous peptide recovered. As with 'hot' plasma, the mobile phase according to Fehder was used (Appendix D). Table 2.8 shows the percentage recovery for SP in plasma and saliva for the different columns.

**Table 2.8:** Percentage recovery for SP in plasma and saliva for different C18 SPE columns

| Manufacturer | SPE column | Sample content | SP (pmol/L) | Percentage recovery (%) |
|---|---|---|---|---|
| **Plasma** | | | | |
| Isolute | C18 NEC 50 mg | Endogenous | 3.964 | |
| | | With spike | 46.989 | 86.1 |
| Isolute | C18 MF 200 mg | Endogenous | 4.282 | |
| | | With spike | 40.852 | 73.1 |
| Varian Bond-elute | C18 OH 100 mg | Endogenous | 5.887 | |
| | | With spike | 53.392 | 95.0 |
| Phenomenex Strata | C18 M 50 mg | Endogenous | 5.987 | |
| | | With spike | 58.733 | 105.5 |
| Varian Bond-elute | C18 50 mg | Endogenous | 3.232 | |
| | | With spike | 27.380 | 48.3 |

| Manufacturer | SPE column | Sample content | SP (pmol/L) | Percentage recovery (%) |
|---|---|---|---|---|
| **Saliva** | | | | |
| Isolute | C18 NEC 50 mg | Endogenous | 2.716 | |
| | | With spike | 51.509 | 97.6 |
| Isolute | C18 MF 200 mg | Endogenous | 2.874 | |
| | | With spike | 29.843 | 53.9 |
| Varian Bond-elute | C18 OH 100 mg | Endogenous | 2.703 | |
| | | With spike | 51.869 | 98.3 |
| Phenomenex Strata | C18 M 50 mg | Endogenous | 3.297 | |
| | | With spike | 48.373 | 90.2 |
| Varian Bond-elute | C18 50 mg | Endogenous | 2.538 | |
| | | With spike | 50.252 | 95.4 |

SPSEFA5

The Isolute C18 MF yielded generally poor recovery for both plasma and saliva. The Varian Bond-elute C18 showed good recovery for saliva, but was very poor for plasma. The Phenomenex Strata C18 M demonstrated an extraordinary plasma recovery of 105%, and previously 103% total recovery with 'hot' plasma. This was deemed unsatisfactory as peptide appeared to be 'manufactured' by the columns, i.e. more was detected than was originally spiked. After the Phenomenex column, the Isolute C18 NEC and Varian C18 OH gave the best recoveries.

### 2.3.3 Comparison between different mobile phases

Although the mobile phase according to Fehder was used for initial SP recovery experiments and appeared to be effective, it performed far less well with NKA. Three C18 SPE columns each were tested with plasma and saliva spiked with 100 pmol/L NKA standard. To assess recovery, plasma and saliva from the same pool, but without any addition of standard, were also extracted to obtain concentrations of endogenous peptide recovered. Table 2.9 shows the percentage recovery for NKA in plasma and saliva for the different columns.

**Table 2.9:** Percentage recovery for NKA in plasma and saliva for different C18 SPE columns using the Fehder mobile phase

| Manufacturer | SPE column | Sample content | NKA (pmol/L) | Percentage recovery (%) |
|---|---|---|---|---|
| **Plasma** | | | | |
| Isolute | C18 NEC 50 mg | Endogenous | 8.420 | 5.0 |
| | | With spike | 12.620 | |
| Varian Bond-elute | C18 OH 100 mg | Endogenous | 5.749 | 19.1 |
| | | With spike | 24.262 | |
| **Saliva** | | | | |
| Isolute | C18 NEC 50 mg | Endogenous | 52.812 | 16.1 |
| | | With spike | 63.640 | |
| Varian Bond-elute | C18 OH 100 mg | Endogenous | 40.705 | 60.5 |
| | | With spike | 97.107 | |

NKASEFA1

This was disappointing as both SP and NKA peptides needed to be extracted during a single extraction procedure. The Fehder mobile phase obviously worked well for SP but not for NKA. A new mobile phase needed to be investigated. The extraction procedure was therefore repeated with the mobile phase according to the Peninsula kit insert (Appendix E). Results are shown in Table 2.10.

**Table 2.10:** Percentage recovery for NKA in plasma and saliva for different C18 SPE columns using the Peninsula mobile phase

| Manufacturer | SPE column | Sample content | NKA (pmol/L) | Percentage recovery (%) |
|---|---|---|---|---|
| **Plasma** | | | | |
| Isolute | C18 NEC 50 mg | Endogenous | 14.225 | 85.1 |
| | | With spike | 99.292 | |
| Varian Bond-elute | C18 OH 100 mg | Endogenous | 19.561 | 42.0 |
| | | With spike | 61.520 | |
| **Saliva** | | | | |
| Isolute | C18 NEC 50 mg | Endogenous | 18.230 | 88.1 |
| | | With spike | 106.330 | |
| Varian Bond-elute | C18 OH 100 mg | Endogenous | 23.437 | 84.4 |
| | | With spike | 107.820 | |

NKASEFA2

The Peninsula mobile phase produced much higher NKA recovery than the Fehder mobile phase. The effect of the Peninsula mobile phase on SP recovery was therefore also tested using 'cold' plasma spiked with 50 pmol/L of SP standard. Results are shown in Table 2.11.

**Table 2.11:** Percentage recovery for SP in plasma and saliva for different C18 SPE columns using the Peninsula mobile phase

| Manufacturer | SPE column | Sample content | SP (pmol/L) | Percentage recovery (%) |
|---|---|---|---|---|
| **Plasma** | | | | |
| Isolute | C18 NEC 50 mg | Endogenous | 2.468 | 71.5 |
| | | With spike | 38.226 | |
| Varian Bond-elute | C18 OH 100 mg | Endogenous | 2.249 | 68.9 |
| | | With spike | 36.689 | |
| **Saliva** | | | | |
| Isolute | C18 NEC 50 mg | Endogenous | 7.059 | 82.7 |
| | | With spike | 48.425 | |
| Varian Bond-elute | C18 OH 100 mg | Endogenous | 10.399 | 89.2 |
| | | With spike | 54.987 | |

SPSEFA6

The Peninsula mobile phase was therefore deemed to be the most suitable for SPE of both SP and NKA from the same sample. With the Peninsula mobile phase, the Isolute C18 NEC column produced a higher percentage recovery compared with the Varian Bond-elute C18 OH. The Isolute C18 NEC column, coupled with the Peninsula mobile phase, was therefore used for all subsequent sample extractions.

## 2.4 DISCUSSION

Peptide extraction was found to be required for the analysis of SP as the 'recovery' of SP was in excess of 100% with non-extracted samples (Section 2.2). This indicated that the antibody was cross-reacting with a matrix component. Furthermore, serial dilution of non-extracted plasma and saliva samples did not yield a serial reduction in measured SP concentration, again indicating that cross-reactivity with a matrix component was occurring (Section 2.1). Finally, the measured SP concentration in non-extracted plasma samples was four times higher than extracted plasma samples, and in non-extracted saliva samples was almost twice as high as extracted saliva samples (Section 2.2). This was in accordance with findings published by Joyce *et al* [192], who discovered that unextracted plasma and synovial fluid gave higher measurements than extracted samples, and queried the accuracy of other published reports on plasma SP variations with clinical conditions where sample extraction had not been performed.

The Yanaihara liquid phase extraction procedure seemed promising initially, with relatively high recoveries of 81% for plasma SP and 97% for saliva SP using 'hot' plasma and saliva (Section 2.2.2). It was identified that the larger losses for plasma SP seemed to occur in the protein precipitates generated by the extraction procedure. The liquid phase extraction procedure was then repeated with 'cold' plasma and saliva, as this would test the compatibility of the post-extraction matrix with the radioimmunoassay. Plasma SP recovery was maintained at 88%, but recovery with saliva SP was much poorer at 68% (Section 2.2.3). Hence, solid phase extraction was investigated as an alternative.

The first stage in the research of solid phase extraction for SP was to determine which type of column was best for the peptide. C18 columns were found to be more efficient than C8 columns at extracting the peptide (Section 2.3.1), and in particular, the Isolute C18 NEC and Varian Bond-elute C18 OH columns yielded the highest recoveries (Section 2.3.2). The next stage was to find a mobile phase that would facilitate good extraction of SP from the plasma and saliva samples by the columns, and would then finish by being able to elute the extracted peptide completely from the columns. Commercially available kits exist for SP radioimmunoassay, but the current investigations have shown that extraction procedures described in the kits do not always produce good recoveries. For example, the extraction procedure recommended by Euro-Diagnostica (Appendix C) resulted in good extraction of SP by the C18 column, but the eluant suggested was then incapable of eluting the peptide from the column (Section 2.3.1). The Euro-Diagnostica kit was therefore being marketed with a recommended extraction procedure that would yield negligible SP recovery.

Following this discovery, the Fehder mobile phase (Appendix D) was used, which closely resembled the Euro-Diagnostica mobile phase, apart from the use of acidified acetonitrile instead of non-acidified methanol as the eluant. This change in eluant yielded a much improved recovery of plasma and saliva SP (>85%) from the C18 columns (Section 2.3.2). However, the same mobile phase produced extremely poor recovery of plasma and saliva NKA from the same columns (<60%) (Section 2.3.3). This was disappointing as both SP and NKA needed to be extracted from the same sample during a single extraction procedure.

A third mobile phase, from Peninsula, was therefore tested. The eluant remained as acidified acetonitrile but the rest of the mobile phase was different (Appendix E). The method was slightly modified in that the elution was conducted under gravity only, whereas the original method stated that a light vacuum pressure might be applied to the columns. The Peninsula mobile phase produced a much higher NKA recovery than the Fehder mobile phase (>85%), and a nonetheless satisfactory recovery for SP recovery (>70%) (Section 2.3.3). With the Peninsula mobile phase, the Isolute C18 NEC column also produced better recoveries than the Varian Bond-elute C18 OH column.

As a consequence of the multiple investigations documented above, the final extraction procedure used was the Peninsula mobile phase coupled with the Isolute C18 NEC columns.

# 3. DEVELOPMENT OF SUBSTANCE P AND NEUROKININ A RADIOIMMUNOASSAYS

A radioimmunoassay is based on relatively simple principles. A fixed concentration of antibody is incubated with step-wise increasing concentrations of antigen (standard). Then a fixed concentration of radioactively-labelled antigen (tracer) is added. This binds the residual antibody sites not occupied by the standard, and tracer and standard will compete for available antibody sites until an equilibrium is attained. The bound fraction of antibody is then precipitated out and unbound antigen removed. The bound fraction is measured in a gamma counter and the count of labelled antigen is inversely related to the concentration of standard. From these results, a standard curve is constructed from which unknown antigen values can be calculated.

Commercial radioimmunoassay kits exist for both SP and NKA. However, the IDS/Euro-Diagnostica SP kit requires 2 ml of plasma for the extraction procedure, a small volume for an adult subject, but an unacceptably large volume for many neonates. In-house radioimmunoassays therefore needed to be developed, with increased sensitivities, to measure SP and NKA concentrations from neonatal microsamples.

Materials used are detailed in Appendix A.

## 3.1 CHOICE OF ANTIBODY

Antibody to SP exists from various sources. However, as NKA was also being studied, it was imperative that no cross-reactivity occurred between the two assays. Table 3.1 summarises the cross-reactivity patterns of different SP antibodies as published by their manufacturers. Table 3.2 summarises similar results for NKA.

**Table 3.1:** Cross-reactivity profiles for various commercially-available SP antibodies

| | **Manufacturer** | | | |
|---|---|---|---|---|
| | IDS/Euro-Diagnostica Cat No. A45 Batch SP-2-840530 Rabbit host | Sigma Cat No. S1542 Rabbit host | Assay Designs Cat No. 90018/90118 Host unknown | Cayman Cat No. 583751 Rabbit host |
| **Polypeptide** | **Cross-reactivity (%)** | | | |
| Substance P | 100% | 100% | 100% | .100% |
| SP sulphoxide | 103% | 122% | 85.9% | 93% |
| SP 4-11 | 22% | 175% | 11.7% | 97% |
| SP 7-11 | <0.001% | 0.96% | 5.9% | 30% |
| **Neurokinin A** | **0.001%** | **0.2%** | **0.8%** | **2.7%** |
| Neurokinin B | 0.001% | | 0.2% | 0.04% |
| Kassinin | <0.001% | | | |
| Eledoisin | 0.001% | | 1.5% | 12% |
| Physalaemin | 0.002% | | 75.3% | |
| Bombesin | 0.05% | | | |
| Neuromedin B | <0.001% | | | |
| Neuromedin C | 0.002% | | | |
| Somatostatin | | 0.3% | <0.001% | |

Other SP antibodies exist for immunostaining and immunoblotting rather than use in a radioimmunoassay, e.g. Diasorin, and manufacturers above.

**Table 3.2:** Cross-reactivity profiles for various commercially-available NKA antibodies

| | **Manufacturer** | |
|---|---|---|
| | IDS/Euro-Diagnostica<br>Cat No. A57<br>Batch YS6989<br>Rabbit host | Peninsula<br>Cat No. RIK7359<br><br>Rabbit host |
| **Polypeptide** | **Cross-reactivity (%)** | |
| Neurokinin A | 100% | 100% |
| NKA sulphoxide | 72% | |
| Neurokinin B | 71% | 80% |
| NKB sulphoxide | 73% | |
| Kassinin | 71% | 100% |
| Eledoisin | 60% | |
| **Substance P** | **0.1%** | **0.05%** |
| Physalaemin | 0.1% | 0.02% |
| Bombesin | 0.01% | |
| Neuromedin B | 0.01% | 0% |
| Neuromedin C | <0.001% | |
| ACTH | | 0% |
| Arg-Vasopressin | | 0% |

From the cross-reactivity profiles, it was decided that the SP antibody of choice was from IDS/Euro-Diagnostica and the NKA antibody of choice was from Peninsula. The published cross-reactivities were checked. Cross-reactivity with NKA in the SP assay was 0% and cross-reactivity with SP in the NKA assay was 2%.

## 3.2 Optimisation of antibody concentration

Following the choice of antibody, the optimal antibody concentration was determined for each assay. The more dilute the antibody, the more sensitive the assay at lower peptide concentrations. However, this has to be balanced with the total binding achieved.

Optimal antibody concentration was therefore investigated by running simultaneous assays where the only difference was that of the antibody concentration added to the assay. This was performed separately for SP and NKA. The results are tabulated below (Tables 3.3 and 3.4). Percentage tracer bound over total is indicated by %B/Tot and percentage tracer bound over reference by %B/Ref.

**Table 3.3:** Determination of optimal SP antibody concentration to be added to assay

| Antibody concentration | Total binding (%) | SP standard (pmol/L) | %B/Tot | %B/Ref |
|---|---|---|---|---|
| **1:300** | 45.35 | 0.98 | 45.59 | 100.53 |
| | | 1.95 | 45.95 | 101.32 |
| | | 3.9 | 44.26 | 97.60 |
| | | 7.8 | 42.02 | 92.66 |
| | | 15.6 | 34.56 | 76.21 |
| | | 31.2 | 23.98 | 52.88 |
| | | 62.5 | 14.55 | 32.08 |
| | | 125 | 5.621 | 12.39 |
| | | 250 | 3.208 | 7.07 |
| | | 500 | 1.831 | 4.04 |
| **1:400** | 39.30 | 0.98 | 39.63 | 100.84 |
| | | 1.95 | 38.14 | 97.05 |
| | | 3.9 | 36.57 | 93.05 |
| | | 7.8 | 31.86 | 81.07 |
| | | 15.6 | 25.58 | 65.09 |
| | | 31.2 | 16.5 | 41.98 |
| | | 62.5 | 8.454 | 21.51 |
| | | 125 | 3.473 | 8.84 |
| | | 250 | 1.788 | 4.55 |
| | | 500 | 1.209 | 3.08 |

| Antibody concentration | Total binding (%) | SP standard (pmol/L) | %B/Tot | %B/Ref |
|---|---|---|---|---|
| **1:500** | 33.94 | 0.98 | 32.01 | 94.31 |
| | | 1.95 | 32.1 | 94.58 |
| | | 3.9 | 29.49 | 86.89 |
| | | 7.8 | 26.66 | 78.55 |
| | | 15.6 | 19.05 | 56.13 |
| | | 31.2 | 12.74 | 37.54 |
| | | 62.5 | 5.975 | 17.60 |
| | | 125 | 2.371 | 6.99 |
| | | 250 | 0.9325 | 2.75 |
| | | 500 | 0.1578 | 0.46 |

SPSCDAD

Good differentiation between top and bottom standards was achieved with all SP antibody concentrations. With a SP antibody concentration of 1:300, the assay was insufficiently sensitive to differentiate SP concentrations <3.9 pmol/L. This was improved with a SP antibody concentration of 1:400. Diluting SP antibody further to 1:500 resulted in a lack of differentiation between the lowest two standards. Hence the ideal SP antibody concentration for use in the assay was 1:400.

**Table 3.4:** Determination of optimal NKA antibody concentration to be added to assay

| Antibody concentration | Total binding (%) | NKA standard (pmol/L) | %B/Tot | %B/Ref |
|---|---|---|---|---|
| **1:50** | 40.89 | 1.95 | 40.32 | 98.61 |
| | | 3.98 | 40.08 | 98.02 |
| | | 7.8 | 40.16 | 98.21 |
| | | 15.6 | 37.89 | 92.66 |
| | | 31.2 | 33.99 | 83.13 |
| | | 62.5 | 27.7 | 67.74 |
| | | 125 | 15.01 | 36.71 |
| | | 250 | 6.029 | 14.74 |
| | | 500 | 2.534 | 6.20 |
| | | 1000 | 1.398 | 3.42 |
| **1:75** | 28.25 | 1.95 | 28.42 | 100.60 |
| | | 3.98 | 28.11 | 99.50 |
| | | 7.8 | 26.67 | 94.41 |
| | | 15.6 | 25.28 | 89.49 |
| | | 31.2 | 20.87 | 73.88 |
| | | 62.5 | 14.2 | 50.27 |
| | | 125 | 8.125 | 28.76 |
| | | 250 | 3.761 | 13.31 |
| | | 500 | 1.493 | 5.28 |
| | | 1000 | 1.085 | 3.84 |

| Antibody concentration | Total binding (%) | NKA standard (pmol/L) | %B/Tot | %B/Ref |
|---|---|---|---|---|
| **1:100** | 19.59 | 1.95 | 19.88 | 101.48 |
| | | 3.98 | 18.88 | 96.38 |
| | | 7.8 | 18.5 | 94.44 |
| | | 15.6 | 16.43 | 83.87 |
| | | 31.2 | 13.33 | 68.04 |
| | | 62.5 | 9.154 | 46.73 |
| | | 125 | 5.274 | 26.92 |
| | | 250 | 2.671 | 13.63 |
| | | 500 | 1.4 | 7.15 |
| | | 1000 | 0.6907 | 3.53 |

NKACQC

At all NKA antibody concentrations, good differentiation was achieved between the top and bottom standards. More importantly, differentiation between the lower standards was considered, as it was estimated that concentrations of NKA in infant samples would be in this vicinity, based on published adult concentrations. At a NKA antibody concentration of 1:50, poor differentiation was achieved at NKA concentrations of <7.8 pmol/L. The assay increased in sensitivity for the 1:75 concentration, as adequate differentiation was demonstrated between NKA concentrations of 3.98 and 7.8 pmol/L. This was further improved when NKA antibody concentration was dropped to 1:100. However, at this concentration, total binding dropped to 19.59%. The ideal NKA antibody concentration for use in the assay was therefore 1:75.

## 3.3 OPTIMISATION OF ASSAY AND SAMPLE VOLUMES

The original SP assay used a sample or standard volume of 100 µl, antibody volume of 200 µl and tracer volume of 200 µl. The original NKA assay required sample, antibody and tracer volumes of 100 µl each. Following the determination of optimal antibody concentrations, it was investigated whether similar assay sensitivities could be preserved if the assay volume were to be halved (mini assay). This was preferable as plasma and saliva samples obtained from infants are of a much smaller volume than can be obtained from adults. Also, the same sample needed to be assayed for SP concentration, NKA concentration, and cortisol concentration to yield parallel results. Tables 3.5 and 3.6 show comparisons between normal and mini SP and NKA assays.

**Table 3.5:** Comparison between normal and mini SP assays

| Assay type | Total binding (%) | SP standard (pmol/L) | %B/Tot | %B/Ref |
|---|---|---|---|---|
| **Original assay** | 44.43 | 0.98 | 43.35 | 97.57 |
| | | 1.95 | 42.5 | 95.66 |
| | | 3.9 | 40.05 | 90.14 |
| | | 7.8 | 35.94 | 80.89 |
| | | 15.6 | 29.71 | 66.87 |
| | | 31.2 | 20.28 | 45.64 |
| | | 62.5 | 9.99 | 22.48 |
| | | 125 | 4.877 | 10.98 |
| | | 250 | 2.515 | 5.66 |
| | | 500 | 1.46 | 3.29 |
| **Mini assay** | 43.72 | 0.98 | 43.86 | 100.32 |
| | | 1.95 | 43.02 | 98.40 |
| | | 3.9 | 39.9 | 91.26 |
| | | 7.8 | 36.21 | 82.82 |
| | | 15.6 | 28.8 | 65.87 |
| | | 31.2 | 20.41 | 46.68 |
| | | 62.5 | 11.21 | 25.64 |
| | | 125 | 4.477 | 10.24 |
| | | 250 | 2.152 | 4.92 |
| | | 500 | 2.172 | 4.97 |

SPSCDAD

**Table 3.6:** Comparison between normal and mini NKA assays

| Assay type | Total binding (%) | NKA standard (pmol/L) | %B/Tot | %B/Ref |
|---|---|---|---|---|
| **Original assay** | 18.94 | 1.95 | 18.54 | 97.89 |
| | | 3.98 | 18.03 | 95.20 |
| | | 7.8 | 17.17 | 90.65 |
| | | 15.6 | 15.03 | 79.36 |
| | | 31.2 | 11.8 | 62.30 |
| | | 62.5 | 8.001 | 42.24 |
| | | 125 | 4.686 | 24.74 |
| | | 250 | 2.442 | 12.89 |
| | | 500 | 1.709 | 9.02 |
| | | 1000 | 0.6081 | 3.21 |
| **Mini assay** | 18.69 | 1.95 | 18.37 | 98.29 |
| | | 3.98 | 18.02 | 96.42 |
| | | 7.8 | 17.14 | 91.71 |
| | | 15.6 | 15.23 | 81.49 |
| | | 31.2 | 11.82 | 63.24 |
| | | 62.5 | 8.01 | 42.86 |
| | | 125 | 5.124 | 27.42 |
| | | 250 | 2.784 | 14.90 |
| | | 500 | 1.488 | 7.96 |
| | | 1000 | 0.5333 | 2.85 |

NKACQC

Both mini assays showed drops in degree of sensitivity when compared to the original assays. However, if 100 µl sample volumes were to be used in accordance with the original assays, the majority of the neonatal microsamples would not be sufficient for analyses of SP, NKA and cortisol.

## 3.4 Comparison of Sigma and Peninsula SP standards

As there were different SP standards available, it was important to determine the affinity between various standards within the in-house assay developed. Synthetic SP (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-$NH_2$) was obtained from Peninsula Laboratories Europe Ltd and Sigma Chemical Company. Details of each standard are provided in the table below. It was expected that the Peninsula standard would exhibit better affinity as the antibody used was raised against it.

**Table 3.7:** Comparison between Peninsula and Sigma SP standards

| | **Peninsula Laboratories Ltd** | **Sigma Chemical Company** |
|---|---|---|
| Catalogue no. | 7451 | S6883 |
| Molecular weight | 1347.66 | 1347.63 |
| HPLC purity | 97.8% (TFA buffer system)<br>99.2% ($NaH_2PO_4$ buffer system) | 98% |
| Capillary electrophoresis | Single peak found | Not stated |
| Maldi mass spectrometry | 1348.90 | Not stated |
| Peptide content | 79.4% | Not stated |

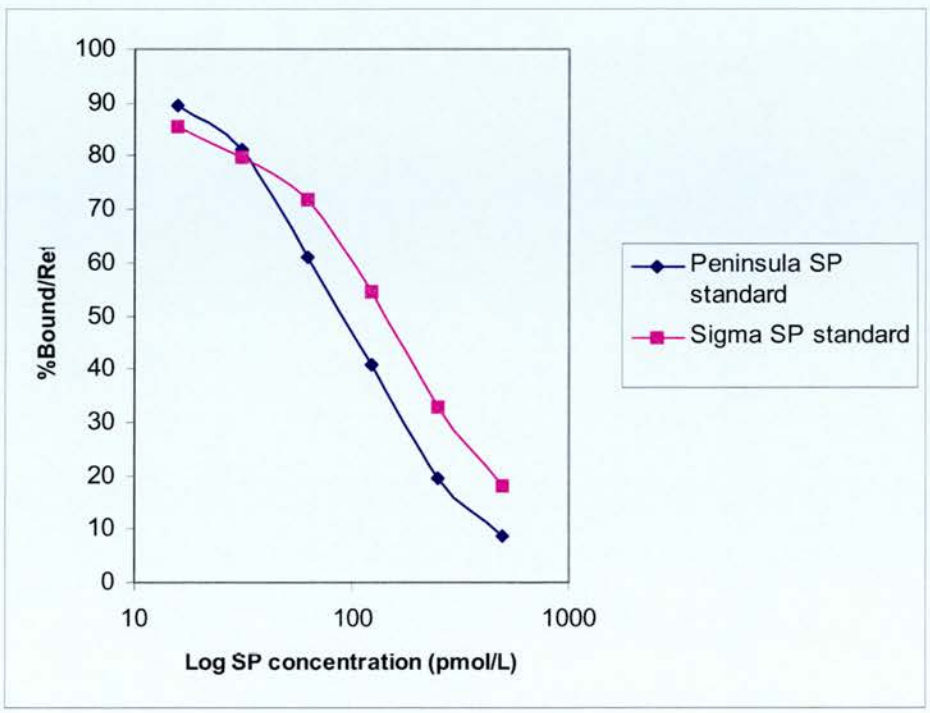
The SP standards were then diluted identically and used in the same assay. Table 3.8 shows the percentage bound over total (%B/Tot) for the respective standards, and Graph 3.1 shows the difference between the standards when plotted as percentage bound over reference (%B/Ref).

**Table 3.8:** %B/Tot for Peninsula versus Sigma SP standards in the same assay

| SP concentration (pmol/L) | Peninsula %B/Tot | Sigma %B/Tot |
|---|---|---|
| 15.6 | 54.38 | 51.97 |
| 31.2 | 49.38 | 48.43 |
| 62.5 | 37.10 | 43.62 |
| 125 | 24.69 | 33.16 |
| 250 | 11.87 | 19.93 |
| 500 | 5.24 | 11.02 |

SPSCC

**Graph 3.1:** Peninsula versus Sigma SP standard curves in the same assay



The experiment was not repeated for NKA. Instead, the Peninsula NKA standard was used routinely as the antibody was also from Peninsula.

## 3.5 STABILITY OF PENINSULA STANDARD

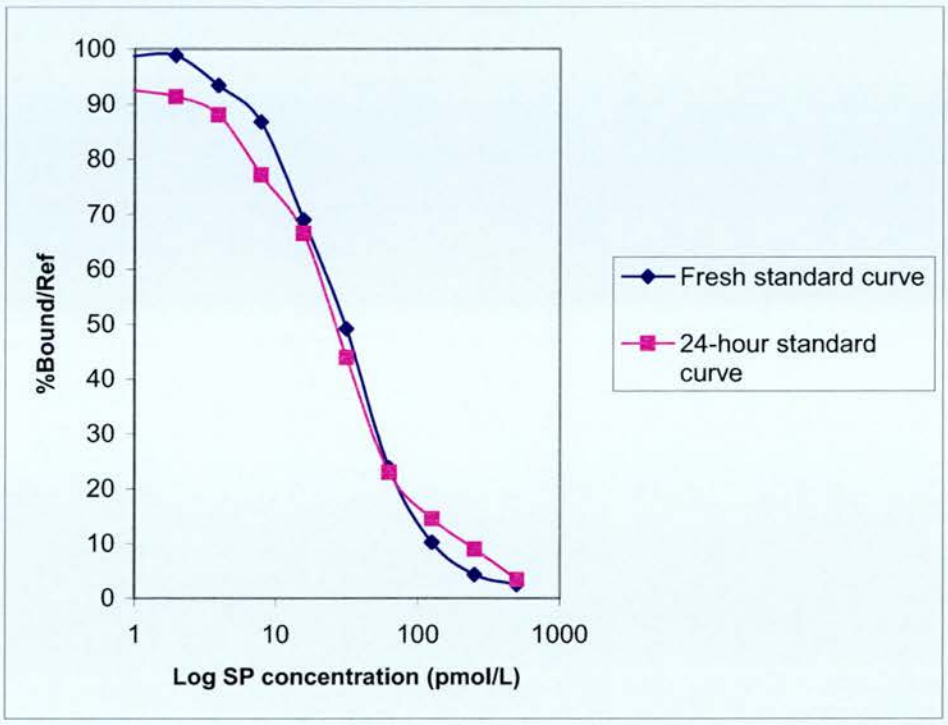
For each assay, the SP standards used were prepared freshly from stock Peninsula SP standard concentrate ($5x10^{-8}$M). The standards were prepared in polypropylene tubes (See Section 4.1). The stability of the diluted standards was assessed by leaving them in the polypropylene tubes for 24 hours at 4°C and repeating the assay to see whether binding was affected (Table 3.9 and Graph 3.2).

**Table 3.9:** Comparison between fresh and 24-hour delayed SP standard curves

| Assay type | Total binding (%) | SP standard (pmol/L) | %B/Tot | %B/Ref |
|---|---|---|---|---|
| **Fresh standards** | 44.18 | 0.98 | 43.61 | 98.71 |
| | | 1.95 | 43.66 | 98.82 |
| | | 3.9 | 41.25 | 93.37 |
| | | 7.8 | 38.33 | 86.76 |
| | | 15.6 | 30.47 | 68.97 |
| | | 31.2 | 21.73 | 49.19 |
| | | 62.5 | 10.52 | 23.81 |
| | | 125 | 4.50 | 10.19 |
| | | 250 | 1.92 | 4.34 |
| | | 500 | 1.10 | 2.49 |
| **24-hour standards** | 45.23 | 0.98 | 41.84 | 92.50 |
| | | 1.95 | 41.32 | 91.36 |
| | | 3.9 | 39.82 | 88.04 |
| | | 7.8 | 34.90 | 77.16 |
| | | 15.6 | 30.08 | 66.50 |
| | | 31.2 | 19.88 | 43.95 |
| | | 62.5 | 10.40 | 22.99 |
| | | 125 | 6.57 | 14.52 |
| | | 250 | 4.06 | 8.98 |
| | | 500 | 1.56 | 3.44 |

SPSCS

**Graph 3.2:** Comparison between fresh and 24-hour delayed SP standard curves




After 24 hours, the diluted SP standards showed a change in binding and a variation in the shape of the standard curve obtained compared to freshly prepared standards. This stressed the importance of using freshly prepared standards for each SP assay performed. The same principle was applied to the NKA assay without repetition of the experiment.

## 3.6 EQUILIBRATED VERSUS DISEQUILIBRATED ASSAY

Radioimmunoassays are based on the principle of competitive binding between peptide (as standard or endogenous) and tracer. When peptide concentrations exist in small amounts, tracer addition can be staggered or delayed to facilitate binding of peptide with antibody in the first instance. This is known as a disequilibrated assay.

The assay for SP was compared in equilibration and disequilibration to see if improved binding of standards, and hence increased sensitivity, could be achieved.

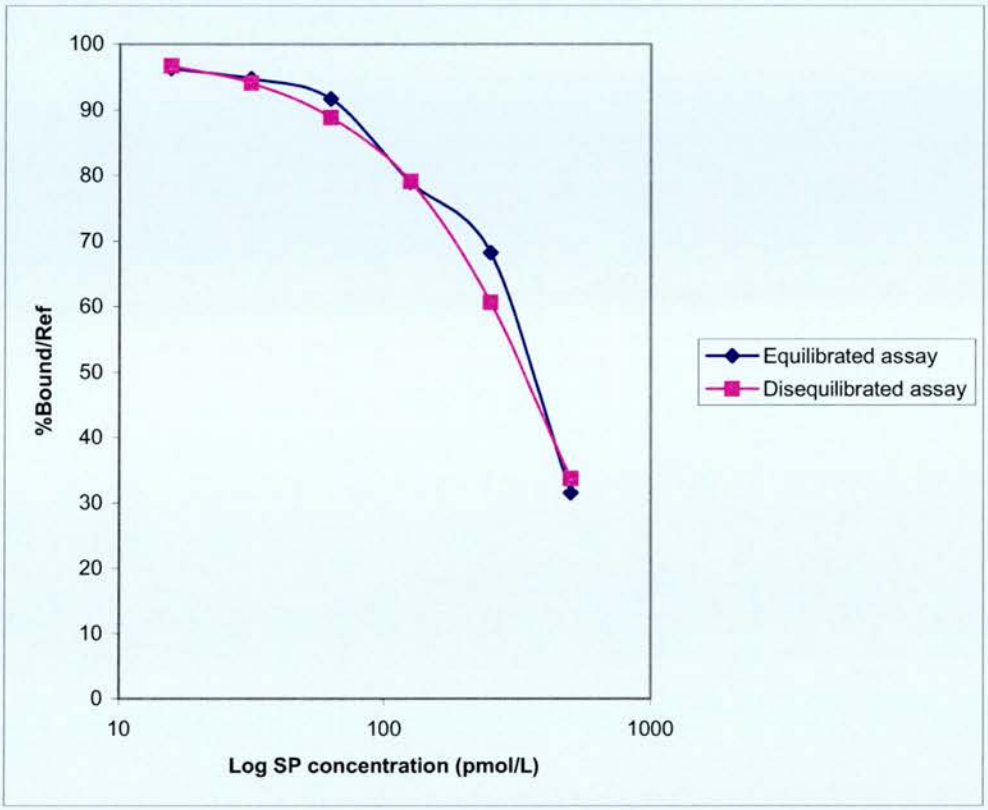
In the equilibrated assay, SP antibody, standard and tracer were mixed together. After 24 hours, a second antibody was added to precipitate the bound fraction out. After a further 24 hours, the supernatants were discarded and the precipitates counted in a gamma counter. In the disequilibrated assay, SP antibody and standard were mixed 24 hours in advance of tracer being added. The rest of the procedure was identical. To allow direct comparison, the assays were done in parallel with the same batch of antibody, tracer and standard (Table 3.10 and Graph 3.3).

**Table 3.10:** Comparison between equilibrated and disequilibrated SP assays

| **SP concentration (pmol/L)** | **%B/Ref** | |
|---|---|---|
| | **Equilibrated assay** | **Disequilibrated assay** |
| 15.6 | 96.27 | 96.72 |
| 31.2 | 94.74 | 94.05 |
| 62.5 | 91.65 | 88.84 |
| 125 | 78.98 | 79.09 |
| 250 | 68.22 | 60.65 |
| 500 | 31.58 | 33.76 |

SPSCDis

**Graph 3.3:** Comparison between equilibrated and disequilibrated SP assays




The results show that delaying the addition of tracer by 24 hours (disequilibrating the assay) yielded a slightly better standard curve. The same principle was also applied to the NKA assay without repetition of the experiment.

## 3.7 EVALUATING DIFFERENT SECOND ANTIBODIES

In the final step of a radioimmunoassay, the fraction of bound primary antibody is separated out to be quantified so that the concentration of analyte can be determined based on an inverse standard curve. A variety of separation methods exist, such as adsorption with activated charcoal, or precipitation with a second antibody or

polyethylene glycol (PEG). Two different second antibody precipitation methods were evaluated, one using donkey anti-rabbit serum with normal rabbit serum (DARS/NRS), and the other using cellulose (Sac-Cel®). The assays were disequilibrated and done in parallel, with the only difference being the type of second antibody used in the final step. Table 3.11 and Graph 3.4 show results for the SP assay, and Table 3.12 and Graph 3.5 show results for the NKA assay.

**Table 3.11:** Comparison between Sac-Cel (two volumes) and DARS/NRS in SP assay

| Assay type | Total binding (%) | SP standard (pmol/L) | %B/Tot | %B/Ref |
|---|---|---|---|---|
| **Sac-Cel assay** | 55.41 | 15.6 | 53.33 | 96.25 |
| (400 µl volume) | | 31.2 | 51.57 | 93.07 |
| | | 62.5 | 47.18 | 85.15 |
| | | 125 | 39.43 | 71.16 |
| | | 250 | 24.50 | 44.22 |
| | | 500 | 11.36 | 20.50 |
| **Sac-Cel assay** | 56.65 | 15.6 | 54.69 | 96.54 |
| (100 µl volume) | | 31.2 | 52.44 | 92.57 |
| | | 62.5 | 48.82 | 86.18 |
| | | 125 | 41.56 | 73.36 |
| | | 250 | 26.07 | 46.02 |
| | | 500 | 14.01 | 24.73 |
| **DARS/NRS assay** | 60.57 | 15.6 | 58.65 | 96.83 |
| | | 31.2 | 56.36 | 93.05 |
| | | 62.5 | 53.05 | 87.58 |
| | | 125 | 45.43 | 75.00 |
| | | 250 | 29.68 | 49.00 |
| | | 500 | 14.77 | 24.39 |

SPSCCellDASP

**Graph 3.4:** Comparison between Sac-Cel (two volumes) and DARS/NRS SP standard curves

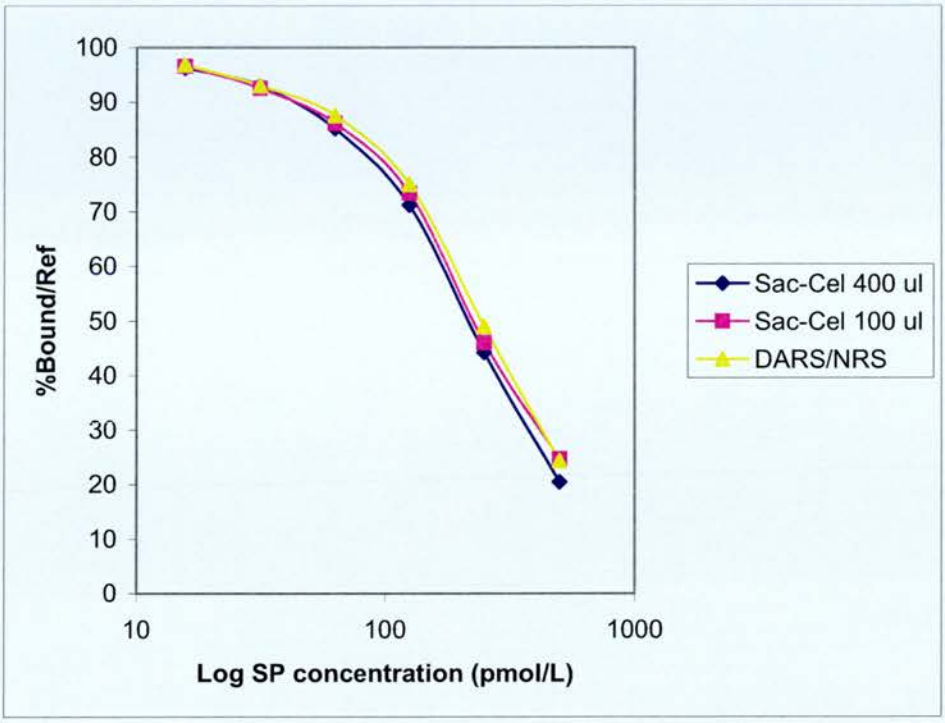
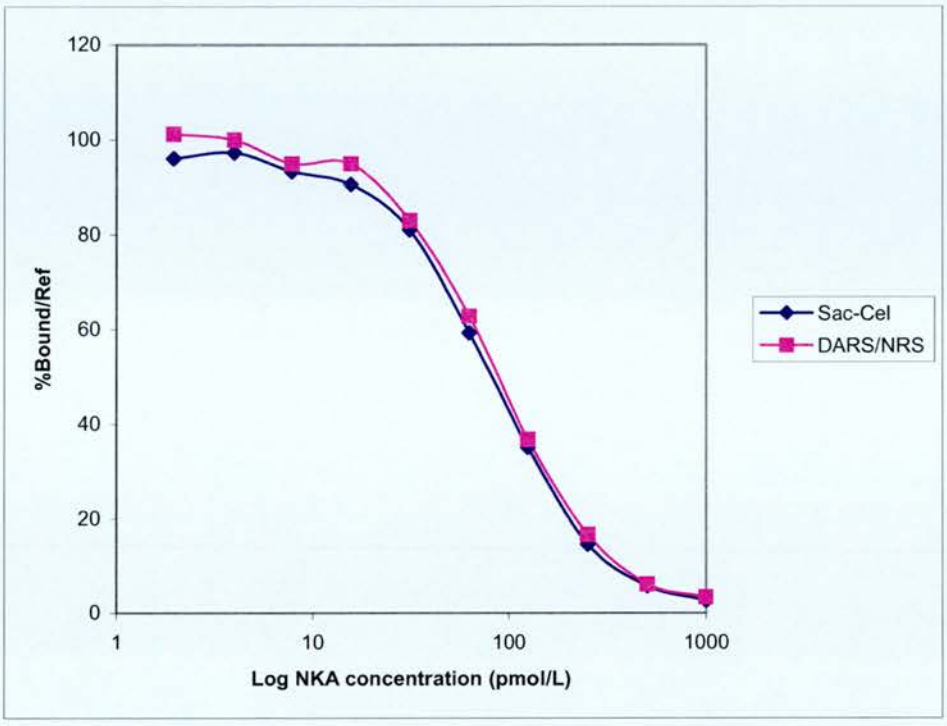


**Table 3.12:** Comparison between Sac-Cel and DARS/NRS in NKA assay

| Assay type | Total binding (%) | NKA standard (pmol/L) | %B/Tot | %B/Ref |
|---|---|---|---|---|
| **Sac-Cel assay** | 47.36 | 1.95 | 45.49 | 96.05 |
| | | 3.98 | 46.07 | 97.28 |
| | | 7.8 | 44.19 | 93.31 |
| | | 15.6 | 42.9 | 90.58 |
| | | 31.2 | 38.38 | 81.04 |
| | | 62.5 | 28.09 | 59.31 |
| | | 125 | 16.61 | 35.07 |
| | | 250 | 6.897 | 14.56 |
| | | 500 | 2.709 | 5.72 |
| | | 1000 | 1.252 | 2.64 |
| **DARS/NRS assay** | 42.72 | 1.95 | 43.24 | 101.22 |
| | | 3.98 | 42.7 | 99.95 |
| | | 7.8 | 40.57 | 94.97 |
| | | 15.6 | 40.57 | 94.97 |
| | | 31.2 | 35.44 | 82.96 |
| | | 62.5 | 26.84 | 62.83 |
| | | 125 | 15.7 | 36.75 |
| | | 250 | 7.11 | 16.64 |
| | | 500 | 2.602 | 6.09 |
| | | 1000 | 1.437 | 3.36 |

NKACQC

**Graph 3.5:** Comparison between Sac-Cel and DARS/NRS NKA standard curves




In both the SP and NKA assays, precipitation with either Sac-Cel or DARS/NRS was equally effective. A volume of 400 µl of Sac-Cel yielded no benefit over a 100 µl volume.

## 3.8 QUALITY CONTROLS

The projected inter-assay variation was determined from six assays each of SP and NKA, at approximately 80%, 50% and 20% binding, i.e. the straight part of the standard curve (Tables 3.13 and 3.14). This showed that inter-assay variation was <8% for the SP assay and <10% for the NKA assay. However, it was subsequently found that the median plasma SP and NKA concentrations in newborn infants were

detected to be lower, i.e. 1.7 pmol/L and 6.0 pmol/L respectively. The inter-assay variation at these low concentrations was calculated to be <12% for the SP assay and <15% for the NKA assay.

**Table 3.13:** Inter-assay quality control values for six SP assays

| **Quality control value (pmol/L)** | **7.8** | **31.2** | **125** | **1.95** |
|---|---|---|---|---|
| | **80%** | **50%** | **20%** | |
| | 8.08439 | 28.59886 | 148.79678 | 1.99785 |
| | 8.97903 | 29.77500 | 129.39935 | 1.47880 |
| | 8.46620 | 28.82507 | 135.37430 | 1.99845 |
| | 7.28155 | 31.23256 | 127.55523 | 1.87751 |
| | 7.93201 | 28.15454 | 125.98616 | 1.77280 |
| | 8.45007 | 29.44108 | 122.32207 | 1.60937 |
| **Mean** | 8.19888 | 29.33785 | 131.57232 | 1.78913 |
| **SD** | 0.57776 | 1.09553 | 9.47384 | 0.21170 |
| **%CV** | **7.0** | **3.7** | **7.2** | **11.8** |

**Table 3.14:** Inter-assay quality control values for six NKA assays

| **Quality control value (pmol/L)** | **15.6** | **62.5** | **250** | **7.8** |
|---|---|---|---|---|
| | **80%** | **50%** | **20%** | |
| | 16.46735 | 59.64672 | 251.45707 | 6.06700 |
| | 12.45740 | 61.29907 | 241.85910 | 9.38516 |
| | 13.55277 | 57.93835 | 273.13021 | 8.73577 |
| | 14.40160 | 67.22630 | 236.20090 | 8.28402 |
| | 15.53646 | 66.68324 | 254.28288 | 7.06139 |
| | 14.44818 | 63.75661 | 269.11434 | 7.98407 |
| **Mean** | 14.47729 | 62.75838 | 254.34075 | 7.91957 |
| **SD** | 1.41587 | 3.77910 | 14.59087 | 1.19328 |
| **%CV** | **9.8** | **6.0** | **5.7** | **15.0** |

## 3.9 Discussion and final SP and NKA radioimmunoassay protocols

As neither SP nor NKA had previously been studied in neonates, the radioimmunoassays required development for use with neonatal microsamples. Ideal antibody concentrations were found to be 1:400 for the SP assay and 1:75 for NKA, with total binding being approximately 39% and 28% respectively (Section 3.2). To permit analyses of both SP and NKA from one neonatal microsample, assay volumes needed to be halved. This was accompanied by inevitable drops in sensitivities of both assays (Section 3.3). However, the quality control results given in Section 3.8 are for the mini assays.

Further investigation of the assay procedure showed that freshly prepared standards were required, as the shape of the standard curve changed when standards were left for 24 hours. This occurred despite the standards being in polypropylene tubes (See Section 4.1) and was possibly due to some interaction between the peptide and tube.

Disequilibration of the assay was performed as this yielded a slightly better standard curve (Section 3.6). It is acknowledged that the difference between the equilibrated and disequilibrated standard curves is mainly seen in two points. Disequilibration of the assay did not however result in any deterioration of the standard curve and was therefore used because the peptide concentrations existed in small amounts. Evaluation of different second antibodies showed equally effective precipitation with either Sac-Cel or DARS/NRS (Section 3.7). Sac-Cel was used in the final assay protocols as the availability was more reliable than that of the DARS/NRS.

Following the development of various aspects of both SP and NKA radioimmunoassays, the final protocols used are detailed in Appendix F.

# 4. SAMPLE COLLECTION

Materials used are detailed in Appendix A.

## 4.1 EVALUATION OF TUBE TYPE

Certain peptides are recognised to have an affinity for polystyrene, hence using polystyrene equipment during sample collection and processing would affect peptide concentrations recovered. The purpose of the following study was to examine the affinity of SP and NKA for polystyrene.

50 µl of stock SP standard ($5x10^{-8}$M) was diluted in 4950 µl assay buffer to yield a top RIA standard of 500 pmol/L. Serial 1:2 dilutions of the top standard down to 0.98 pmol/L were performed to construct a standard curve for RIA. Standards were made up in polypropylene tubes and vortex mixed. Half a volume of each standard (1 ml) was then transferred to an identical polystyrene tube and vortex mixed.

The polystyrene standards were then evaluated in an assay using the polypropylene standards to construct the standard curve. Table 4.1 shows measured SP concentrations of the polystyrene standards against expected SP concentrations.

**Table 4.1:** SP concentrations of polystyrene standards against polypropylene SP concentrations

| Expected SP concentration (pmol/L) | Measured SP concentration (pmol/L) | Percentage recovery (%) |
|---|---|---|
| 0.98 | 0 | 0.0 |
| 1.95 | 0 | 0.0 |
| 3.9 | 1.7816 | 45.7 |
| 7.8 | 6.3452 | 81.4 |
| 15.6 | 12.394 | 79.5 |
| 31.2 | 20.269 | 65.0 |
| 62.5 | 52.207 | 83.5 |
| 125 | 93.877 | 75.1 |
| 250 | 140.23 | 56.1 |
| 500 | 185.15 | 37.0 |

SPLEFA16

Clearly, the use of polystyrene tubes affected the concentration of SP in all the diluted standards. In the lowest two standards, SP was undetectable after only brief contact with polystyrene. This was most likely due to adherence of the small peptide to polystyrene. It was therefore vital that all the materials involved in sample collection, peptide extraction and peptide assay were made of polypropylene, and not polystyrene.

## 4.2 EFFECT OF TIME AND ICE ON SAMPLE INTEGRITY

The effect of time elapsed and use of ice between sample collection and centrifugation/freezing were assessed separately for endogenous and exogenous SP and NKA.

### 4.2.1 Endogenous tachykinin recovery

15 ml blood was obtained from one adult subject by venepuncture and divided into ten aliquots of 1.5 ml each. Aliquots were in polypropylene microtubes containing freeze-dried aprotinin and ethylenediaminetetraacetic acid (EDTA) (50 μl each). Five microtubes of whole blood were kept on wet ice throughout while the other five were at room temperature. At the times specified in the table below, one microtube from each group was spun in a microfuge for 1 minute, the plasma was decanted into a corresponding polypropylene microtube containing freeze-dried aprotinin and EDTA, and then frozen at -20°C.

Tachykinins were extracted from 0.5 ml plasma as described in Appendix E and assayed for SP and NKA. Table 4.2 shows concentrations of endogenous SP and NKA recovered at the various time periods.

**Table 4.2:** Effect of time and ice on endogenous plasma tachykinins

| Time | Substance P (pmol/L) | | Neurokinin A (pmol/L) | |
|---|---|---|---|---|
| (min) | Ice | No ice | Ice | No ice |
| 0 | 12.132 | 10.384 | 27.221 | 26.433 |
| 5 | 13.256 | 10.119 | 28.581 | 27.913 |
| 10 | 13.157 | 10.258 | 25.492 | 28.895 |
| 20 | 14.120 | 8.071 | 35.266 | 33.134 |
| 60 | 14.066 | 5.129 | 30.129 | 23.387 |

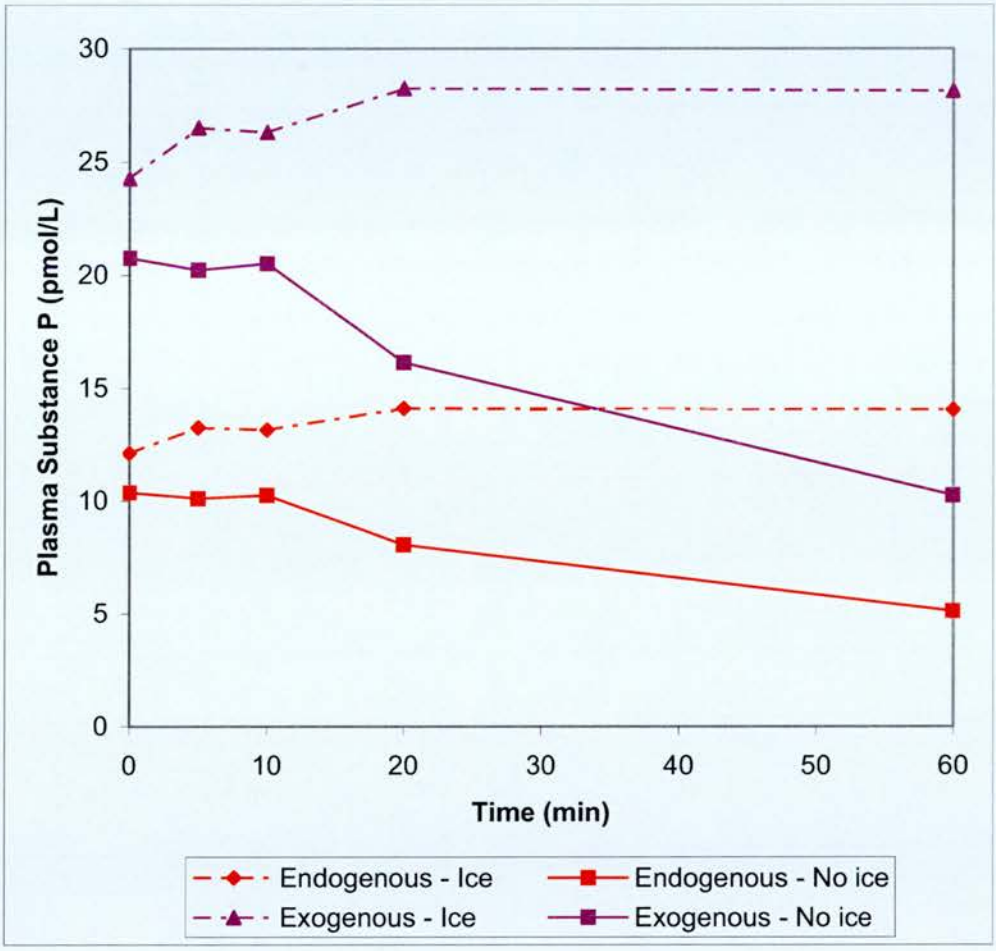
### 4.2.2 Exogenous tachykinin recovery

50 ml blood was obtained from one adult subject by venepuncture and immediately mixed with 25 µl of stock SP standard ($5x10^{-8}$M) and 25 µl of stock NKA standard ($1x10^{-7}$M). Aliquots were prepared as described in Section 4.2.1, and the rest of the experimental procedure followed. Table 4.3 shows concentrations of SP and NKA recovered at the various time points. Graphs 4.1 and 4.2 show the data displayed for SP and NKA respectively.

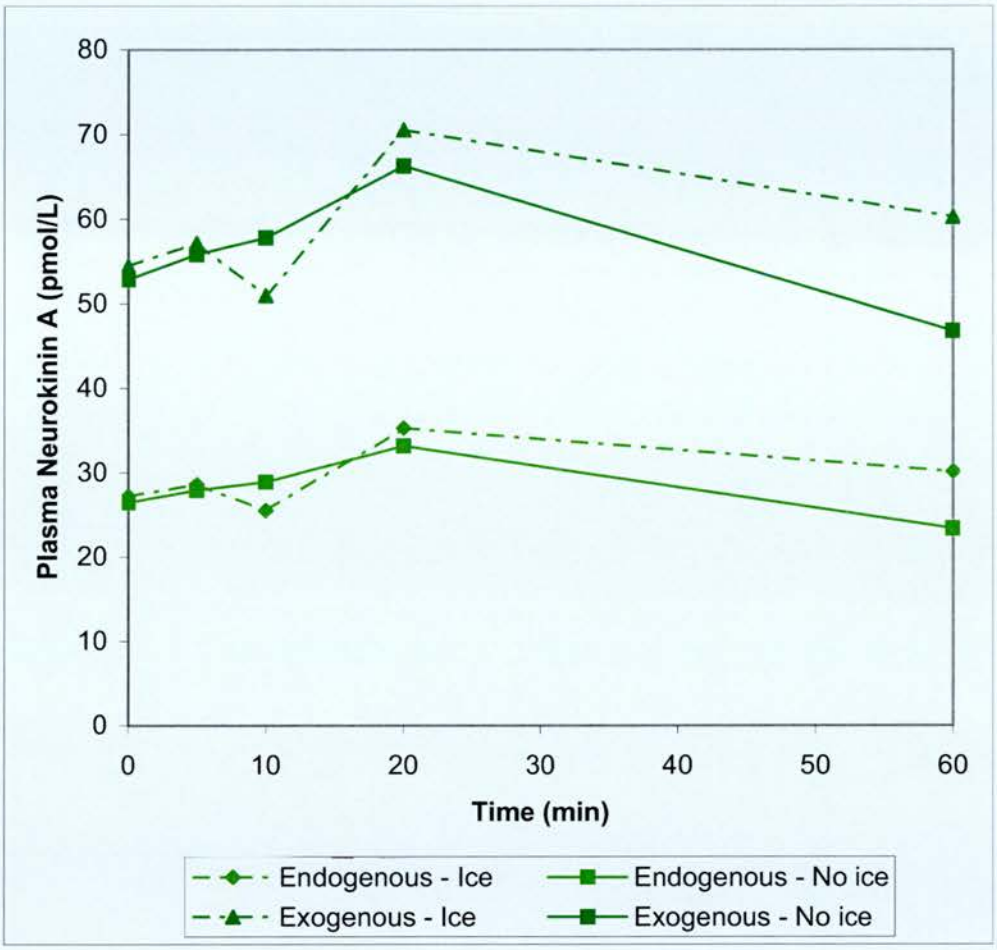
**Table 4.3:** Effect of time and ice on exogenous plasma tachykinins

| **Time** | **Substance P (pmol/L)** | | **Neurokinin A (pmol/L)** | |
|---|---|---|---|---|
| **(min)** | **Ice** | **No ice** | **Ice** | **No ice** |
| 0 | 24.263 | 20.768 | 54.442 | 52.866 |
| 5 | 26.511 | 20.238 | 57.161 | 55.825 |
| 10 | 26.314 | 20.516 | 50.983 | 57.789 |
| 20 | 28.239 | 16.141 | 70.531 | 66.267 |
| 60 | 28.132 | 10.258 | 60.257 | 46.773 |

**Graph 4.1:** Effect of time and ice on endogenous and exogenous SP concentrations *in vitro*



**Graph 4.2:** Effect of time and ice on endogenous and exogenous NKA concentrations *in vitro*




These data show that SP and NKA concentrations remain stable in blood samples that are collected into microtubes containing freeze-dried aprotinin and EDTA, provided they are centrifuged, plasma decanted, and snap-frozen within 10 minutes of collection, whether or not ice is used.

## 4.3 DISCUSSION AND FINAL COLLECTION PROCEDURE

Following the determination of best practice for sample collection, the final sample collection procedure is detailed in Appendix G. It was found that SP concentrations were reduced in assay buffer even after only brief contact with polystyrene (Section 4.1). Care was subsequently taken to ensure that all samples were collected, stored and extracted in polypropylene tubes only. Interestingly, the peptide extraction procedure described by Peninsula (Appendix E) stipulated that the eluant should be collected in a polypropylene tube.

Section 4.2 showed that both endogenous and exogenous concentrations of NKA remained stable in blood samples that were collected into microtubes containing freeze-dried aprotinin and EDTA, as long as the samples were centrifuged, plasma separated, and snap-frozen within 10 minutes of collection. NKA concentrations were stable regardless of whether ice was used or not. Concentrations of SP also remained stable if samples were processed within 10 minutes, but keeping the samples on ice seemed to yield higher overall concentration values of SP, even in the first sample processed immediately (i.e. with no contact time with the ice). The longer the sample spent on ice, the higher the SP concentration measured, whereas the samples not kept on ice showed a natural decline in SP concentration, as would be anticipated from endogenous enzymatic degradation. The increase in SP concentration on ice could possibly be due to chance variation in the blood sample during division into aliquots, or a real effect of ice causing dissociation of SP from albumin or other binding proteins, resulting in increased antibody interaction. Hence, to avoid peptide losses from enzymatic degradation, the final Study Collection

Procedure (Appendix G) stipulated that all samples should be kept on ice during collection, and that the time from start of sample collection to freezing should be no longer than 10 minutes.

The finding of the current study is supported by the work of Joyce *et al*[192], who confirmed that synthetic SP was degraded in a temperature- and time-dependent manner when incubated with human plasma or synovial fluid. Using a cocktail of protease inhibitors, this degradation was prevented without the use of ice.

# 5. METHODOLOGY FOR SUBSEQUENT CLINICAL STUDIES

## 5.1 AIMS

The development of in-house extraction and radioimmunoassay procedures for SP and NKA (Chapters 3 and 4) allowed for the investigation of the tachykinins in neonatal samples to be undertaken. The overall aim of the current clinical study was to examine whether SP, NKA and cortisol could be used as markers of persistent pain in neonates.

Specific aims were:

i) To establish baseline concentrations of plasma and salivary SP and NKA in normal healthy newborns and detect any variation between different gestational age groups and postnatal ages.

ii) To investigate the relationship between plasma and salivary concentrations of SP, NKA, and cortisol.

iii) To observe how conditions which are likely to result in persistent pain, e.g. meningitis, intraventricular haemorrhage (grades III and IV), necrotising enterocolitis (NEC) and surgery, affect SP and NKA concentrations, and whether analgesia makes a difference to the concentrations.

iv) To determine whether assisted ventilation is a cause of persistent pain or non-painful stress to otherwise healthy but premature neonates. At the time of this study, it was possible to capitalise on the Edinburgh Neonatal Unit's

involvement in the international NEOPAIN study[193], which was a randomised double-blinded controlled trial investigating the routine use of morphine infusions versus placebo to sedate ventilated preterm infants. SP and NKA concentrations were assayed in these groups and compared between them as well as with non-ventilated controls.

v) Comparison was made between Premature Infant Pain Profile (PIPP) scores and physiological markers of pain and distress (heart rate and its variability) in ventilated versus non-ventilated infants. I also examined any correlation between these markers and SP or NKA concentrations.

The research hypothesis was that a rise in SP or NKA concentrations is a manifestation of persistent pain in neonates.

## 5.2 MATERIALS (SEE APPENDIX A)

## 5.3 STUDY DESIGN

### 5.3.1 Study cohort selection

The study was a prospective case-controlled observational cohort pilot study, conducted at the Level III Neonatal Unit of the Simpson Memorial Maternity Pavilion, at the Royal Infirmary of Edinburgh. The study period was from July 2000 to December 2001. There was no interventional group and hence no randomisation or blinding was possible. Ethical approval was obtained from the Lothian Research Ethics Committee (Ref: 1702/99/6/48) and the Lothian University Hospitals NHS Trust Research and Development Office (Ref: 99/19/03). Infants were enrolled with

written informed consent from parents, supported by an information sheet (Appendix H). They were recruited in the first 24 hours of life. Inclusion criteria were (i) normal healthy newborns of different gestational ages not requiring ventilatory or other invasive support, (ii) newborns of any gestational age with a 'painful' condition, and (iii) newborns of any gestational age requiring ventilatory support with no other apparent painful condition or intervention. Neonates who developed NEC or meningitis or who underwent surgery were recruited within 24 hours of the illness developing. Exclusion criteria were chromosomal abnormalities, periventricular leucomalacia and seizures.

The study cohort comprised 174 infants with a gestation range of 23 to 42 weeks. Gestational scatter is shown in Graph 5.1. Table 5.1 shows the demographic summary for the entire cohort, and Table 5.2 shows perinatal factors within different gestational age groups.

**Graph 5.1:** Gestational scatter of all infants in study cohort

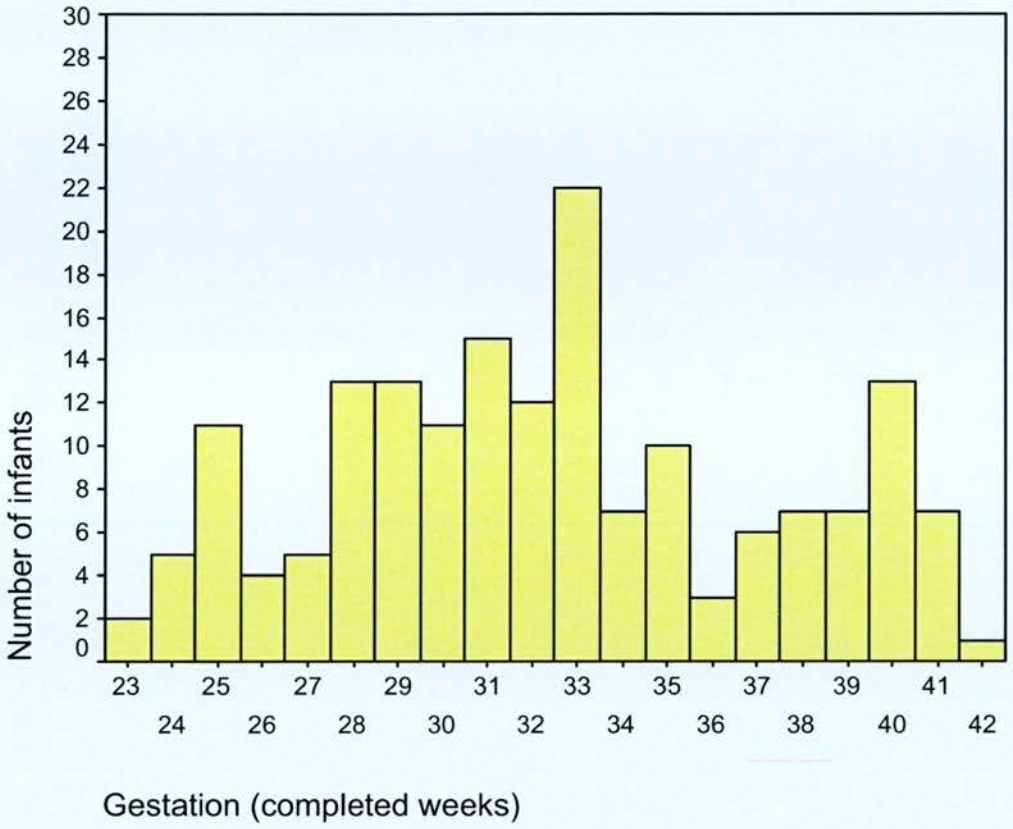



**Table 5.1:** Demographic summary for all study infants

| Demographic factors | N | Mean ± 2SD | Median (Range) |
|---|---|---|---|
| Gestation (weeks) | 174 | 32.4 ± 9.7 | 32 (23 – 42) |
| Birth weight (grams) | 174 | 1990.4 ± 2035.6 | 1845 (590 – 4610) |

**Table 5.2:** Perinatal factors within different gestational age groups

| | **Gestational age group** | | | |
|---|---|---|---|---|
| | **≤ 28 weeks** | **29 – 32 weeks** | **33 – 36 weeks** | **≥ 37 weeks** |
| Number of infants | 40 | 51 | 42 | 41 |
| Median birth weight (grams) (Range) | 822.5 (590 – 1320) | 1490 (635 – 3000) | 2055 (1324 – 2830) | 3342 (1640 – 4610) |
| Caesarian section | 45% | 70.6% | 66.7% | 46.3% |
| Labour | 80% | 64.7% | 54.8% | 80.5% |
| Antenatal steroids | 87.5% | 91.7% *(N=48)* | 68.5% *(N=38)* | 2.4% |
| Median Apgar at 1 min (Range) | 5.5 (1 – 9) *(N=38)* | 7 (2 – 9) *(N=50)* | 8.5 (4 – 9) | 9 (3 – 9) |
| Median Apgar at 5 min (Range) | 8 (4 – 9) *(N=38)* | 9 (4 – 9) *(N=50)* | 9 (6 – 10) | 9 (5 – 9) |
| Mean cord pH | 7.25 *(N=33)* | 7.26 *(N=38)* | 7.27 *(N=28)* | 7.27 *(N=20)* |

*5.3.1.1 NEOPAIN subgroup infants*

Thirty-four infants ≤ 32 weeks' gestation were concurrently enrolled into the NEOPAIN study. All infants on a NEOPAIN study drug infusion (21 morphine, 13 placebo) were ventilated but had no other known painful condition. Gestation, birth weight and perinatal factors were similar in both study drug groups (Table 5.3). Statistics were performed using the unpaired *t* test for parametric data, and the Mann-Whitney U test for non-parametric data.

**Table 5.3:** Demographic comparison of morphine and placebo groups of infants enrolled in the NEOPAIN study

| **Demographic factors** | **Morphine N=21** | **Placebo N=13** | **P value** |
|---|---|---|---|
| Mean gestation (weeks) | 27.3 | 26.6 | 0.4 |
| Median birth weight (g) | 1050 | 870 | 0.6 |
| Median Apgar at 1 min | 6 | 5 | 0.4 |
| Median Apgar at 5 min | 8 | 8 | 0.7 |
| Mean cord pH | 7.25 | 7.23 | 0.5 |
| Caesarian section | 11 (52%) | 6 (42%) | 1.0 |
| Labour | 17 (81%) | 11 (85%) | 1.0 |
| Antenatal steroids | 17 (81%) | 12 (92%) | 0.6 |

*5.3.1.2 Infants with pain*

Of 174 infants, 19 were believed to be suffering persistent pain, e.g. due to surgery, NEC, severe intraventricular haemorrhage (IVH), meningitis (Table 5.4). For simplicity, these infants will henceforth be referred to as being 'in pain'. Three infants had both NEC and surgery. Five of these infants were enrolled from the surgical Neonatal Unit at the Royal Hospital for Sick Children, Edinburgh. The gestational scatter for infants in pain is shown in Graph 5.2. Nineteen controls matched for gestation and ventilatory status were identified from the cohort. Table 5.5 shows the demographic characteristics of the infants in pain and their controls.

**Table 5.4:** Conditions believed to be a source of persistent pain

| Cause of pain | Number of infants affected |
|---|---|
| Surgery (laparotomy or thoracotomy) | 7 |
| Chest drain | 6 |
| Severe IVH (Grades III or IV) | 4 |
| Necrotising enterocolitis | 3 |
| Meningitis | 2 |

**Graph 5.2:** Gestational scatter of infants in pain

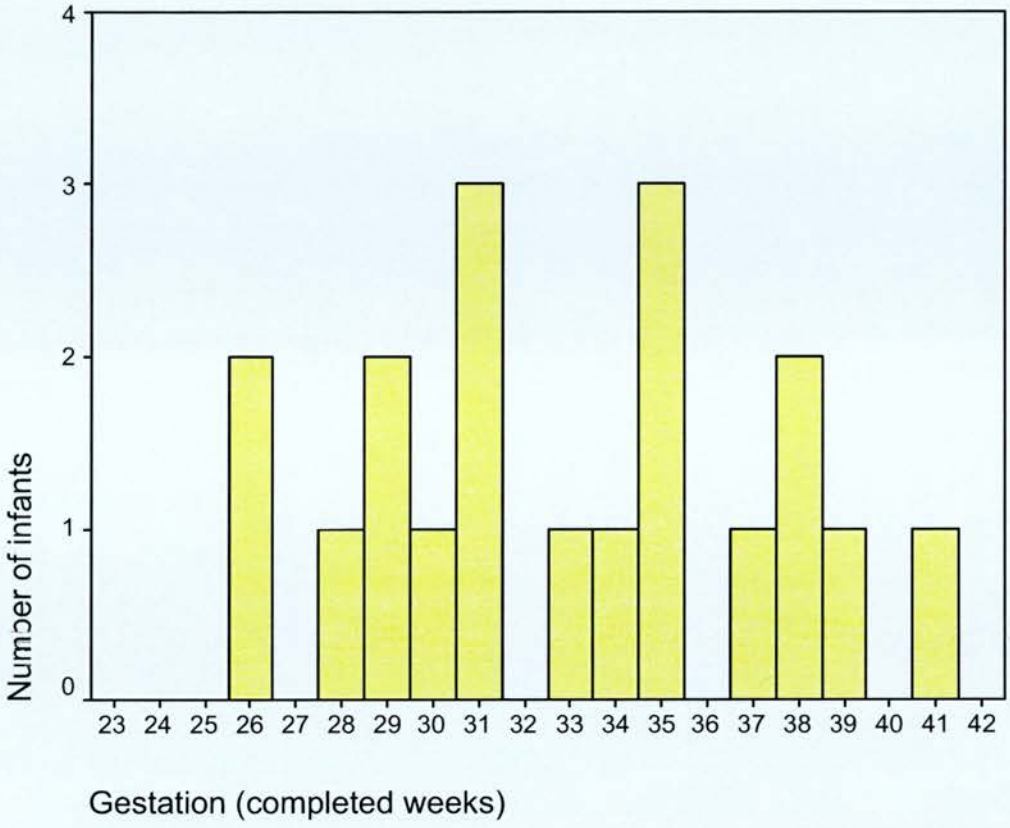



**Table 5.5:** Characteristics of infants in pain versus controls

| | **Infants in pain** | **Controls** |
|---|---|---|
| Gestation (weeks) | 26-41 (median 33) | 25-41 (median 33) |
| Birth weight (g) | 640-3480 (mean 2000) | 590-3770 (mean 2070) |
| Male:female | 10:9 | 9:10 |

### 5.3.2 Clinical sampling procedure

Once-daily, contemporaneous plasma and saliva samples were obtained on days 1, 2, 3, 7 and 14 from the study infants, with the exception of those in pain. They had up to five daily samples taken during the course of their painful condition.

Sampling was opportunistic at a time when the infant required a clinically indicated blood test. Prior to any handling, study infants were scored at rest using the Premature Infant Pain Profile (PIPP) (See Section 1.4.1.4). A second PIPP score was obtained during saliva suction as a standard intervention. If venepuncture was required for the blood sample, then a third PIPP score was obtained to measure the response to the pain stimulus. The Neonatal Unit at the Simpson Memorial Maternity Pavilion has computer facilities for continuous trend monitoring of physiological parameters such as heart rate (See Section 5.3.4). The time of study sampling was recorded from the trend monitoring system. A 30 minute period of quiet rest prior to the handling was later identified and heart rate data subsequently retrieved and used for analysis of heart rate variability.

Saliva sampling was performed at least 30 minutes after the end of a feed to avoid contamination of the saliva sample with mother's breast milk. This was important as human milk is known to have high concentrations of SP ($129.2 \pm 27$ ng/L)[194]. Saliva was acquired by gentle suctioning of oral secretions at a pressure of 12 $cmH_2O$ in a method identical to that routinely used to clear secretions in ventilated neonates. A period of no more than 5 minutes was spent obtaining saliva, which was collected directly onto freeze-dried aprotinin (50 μl) in a suction trap. The sample was then

frozen immediately at -20°C. The Sarstedt Salivette® system was not used in the infants as the Salivette rolls were too large, and there have been reports in the literature of the device interfering with salivary hormone analyses, e.g. 17-hydroxyprogesterone[195].

Immediately after saliva collection, a blood sample was obtained from an indwelling arterial line whenever possible, or a venepuncture concomitant with blood samples required for clinical care. No additional venepunctures or heel lances were performed solely for the purpose of the study. An extra 1 ml of blood was collected into a polypropylene microtube containing freeze-dried aprotinin and EDTA (50 μl each). The whole blood was mixed gently to avoid haemolysis and spun in a microfuge for 1 minute. Plasma was decanted into another identical microtube and frozen immediately. The time taken for separation and freezing of plasma samples did not exceed 10 minutes (Appendix G). Data were then recorded on the clinical state of the infant and current treatment.

Reasons for not obtaining all data on all designated study days were:

- Infant discharged home or transferred to another hospital
- Infant deemed too clinically unstable for handling
- Clinically required blood samples already taken by clinical staff
- Heart rate monitoring not required

### 5.3.3 Sample analysis

Plasma and saliva samples were stored at -70°C until analysis. Peptides were then extracted using a solid phase extraction procedure and SP and NKA quantified using in-house radioimmunoassays, developed as described in Chapter 3. Cortisol was measured using a previously developed direct in-house immunoassay.

### 5.3.4 Heart rate data extraction and calculation of heart rate variability

Heart rate was recorded using either electrocardiograph (ECG) leads or by means of continuous intra-arterial blood pressure waveform monitoring. The cotside system used was the Siemens Infinity SC7000 modular monitoring series. Both ECG and arterial heart rates were produced as analog output with ≤ 25 ms delay.

#### *5.3.4.1 ECG data acquisition*

| | | |
|---|---|---|
| Display: | Up to 12 leads | |
| Neonatal: | I, II, III, aVR, aVL, aVF, V, V+ | |
| Measuring range: | 15 – 300 /min | |
| Accuracy: | ± 2 /min or ± 1% (whichever is greater) | |
| QRS detection: | Amplitude: | 0.5 – 5.0 mV |
| | Duration: | 40 – 120 ms |

*5.3.4.2 Intra-arterial blood pressure waveform acquisition*

| | |
|---|---|
| Measuring method: | Resistive strain gauge transducer |
| Display resolution: | 1 mmHg |
| Measuring range: | -50 to 400 mmHg |
| Accuracy: | ± 1 mmHg or ± 3% exclusive of transducer (whichever is greater) |
| Zero balance range: | ± 200 mmHg |
| Transducer specifications: | Siemens-approved transducers with a resistance of 200 – 300 Ω and an equivalent pressure sensitivity of 5 μV/V/mmHg ± 10% |

*5.3.4.3 The Badger patient data management system*

Continuous trend data were recorded by the Badger System® (www.clevermed.com), a real-time intensive care physiological monitoring and patient data management system. The Badger System downloads and stores second-by-second readings of physiological parameters produced by the Siemens monitoring system, including heart rate, intra-arterial blood pressure, oxygen saturation, respiratory rate and temperature (Figure 5.1). A 30 minute period of quiet rest prior to study handling was identified for each infant. This period yielded 1800 second-by-second readings of heart rate, which were extracted using a programme called BadgerView®. These readings were then used for calculation of mean heart rate and heart rate variability. Heart rate variability was computed as one standard deviation (1SD) of the mean heart rate (See Section 1.4.2.1).

**Figure 5.1:** Screenshot of the Badger System


(Used with permission)

### 5.3.5 Data analysis

All data were analysed using SPSS for Windows Version 11.0. Data that were not normally-distributed were tested using the Mann-Whitney U test. Normally-distributed or log-transformed data were tested using an independent-samples *t* test. Spearman correlation coefficients (r) are quoted with two-tailed tests of significance, as the hormone data are not normally distributed. Pearson correlation coefficients (also r) are used when data are of a normal distribution.

# 6. CLINICAL STUDY: BASELINE CONCENTRATIONS OF PLASMA SUBSTANCE P, NEUROKININ A AND CORTISOL IN NEONATES AND FACTORS AFFECTING THEM

## 6.1 INTRODUCTION AND AIMS

Before embarking on a study to investigate how SP and NKA may vary with pain in neonates, it was important to establish baseline concentrations in neonates across different gestational age groups, and to look for any natural variation in SP and NKA concentrations with postnatal age or perinatal factors. These would be possible confounding factors that would need to be taken into consideration when analysing subsequent studies.

The specific aims here were:

i) To establish baseline concentrations of plasma and salivary SP and NKA in normal healthy newborns and detect any variation between different gestational age groups and postnatal ages.

ii) To investigate the relationship between plasma and salivary concentrations of SP, NKA, and cortisol.

iii) To see if perinatal factors affect SP and NKA concentrations in newborn infants.

The methods used are described in Chapter 5. Infants in pain were excluded from all analyses for the description of baseline concentrations (Sections 6.1 to 6.3).

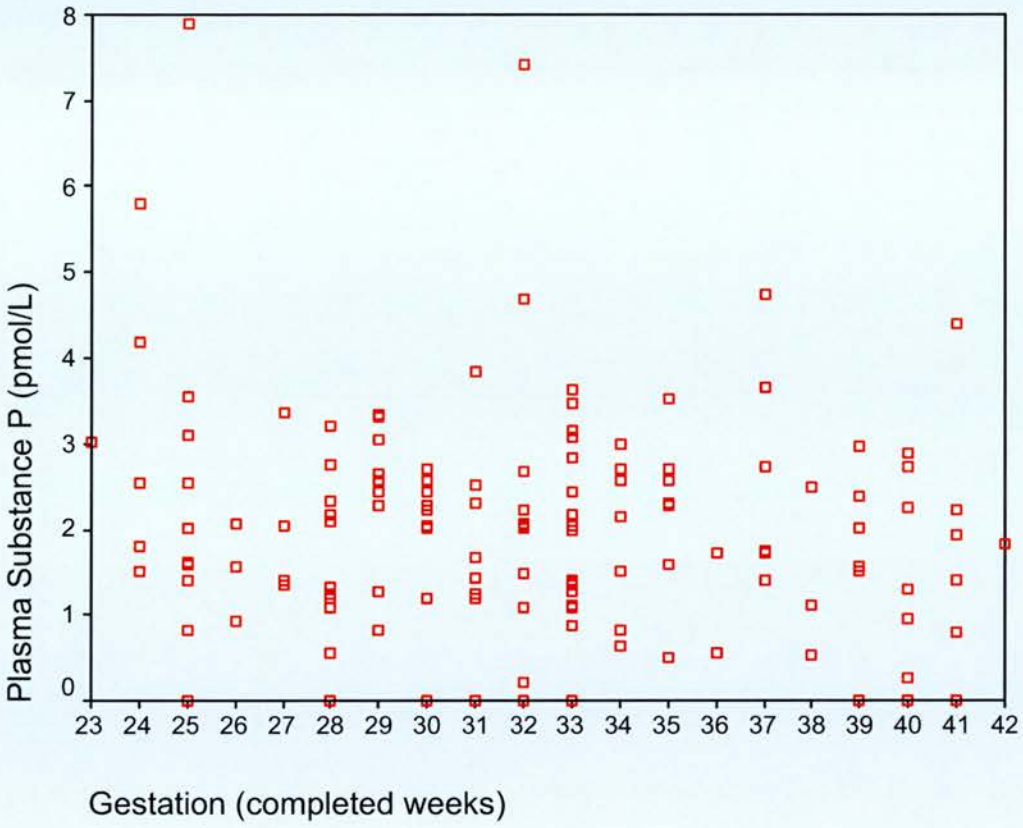
## 6.2 BASELINE CONCENTRATIONS OF PLASMA SP IN NEONATES

Plasma SP concentrations in neonates ranged from 0.0 to 11.2 pmol/L (median 1.7 pmol/L). Fifty-five of 485 results yielded a result of 0.0 pmol/L (11%).

### 6.2.1 Variation of plasma SP with gestation

There was no significant correlation between plasma SP concentrations and gestation ($r = -0.12$, $P = 0.2$) (Graph 6.1) and birth weight ($r = -0.09$, $P = 0.3$).

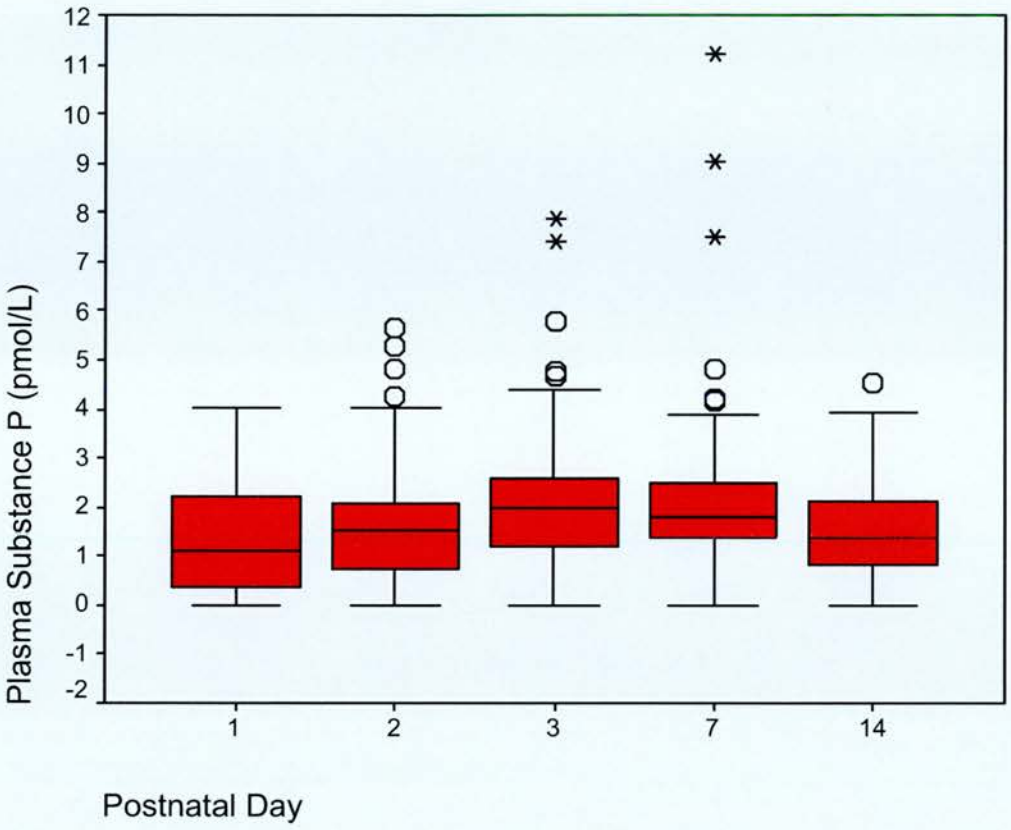
**Graph 6.1:** Variation of plasma SP with gestation



### 6.2.2 Variation of plasma SP with postnatal age

Postnatally, there was a gradual rise in median plasma SP during the first three days which decreased again by day 14 (Graph 6.2). Using a one-way between subjects analysis of variance (ANOVA), this postnatal variation of plasma SP was found to be statistically significant ($F(4, 480) = 5.189$, $P < 0.001$). This pattern was more apparent in preterm infants ≤ 32 weeks' gestation (Graph 6.3) (ANOVA: $F(4, 314) = 6.146$, $P < 0.001$).

**Graph 6.2:** Variation of plasma SP with postnatal age

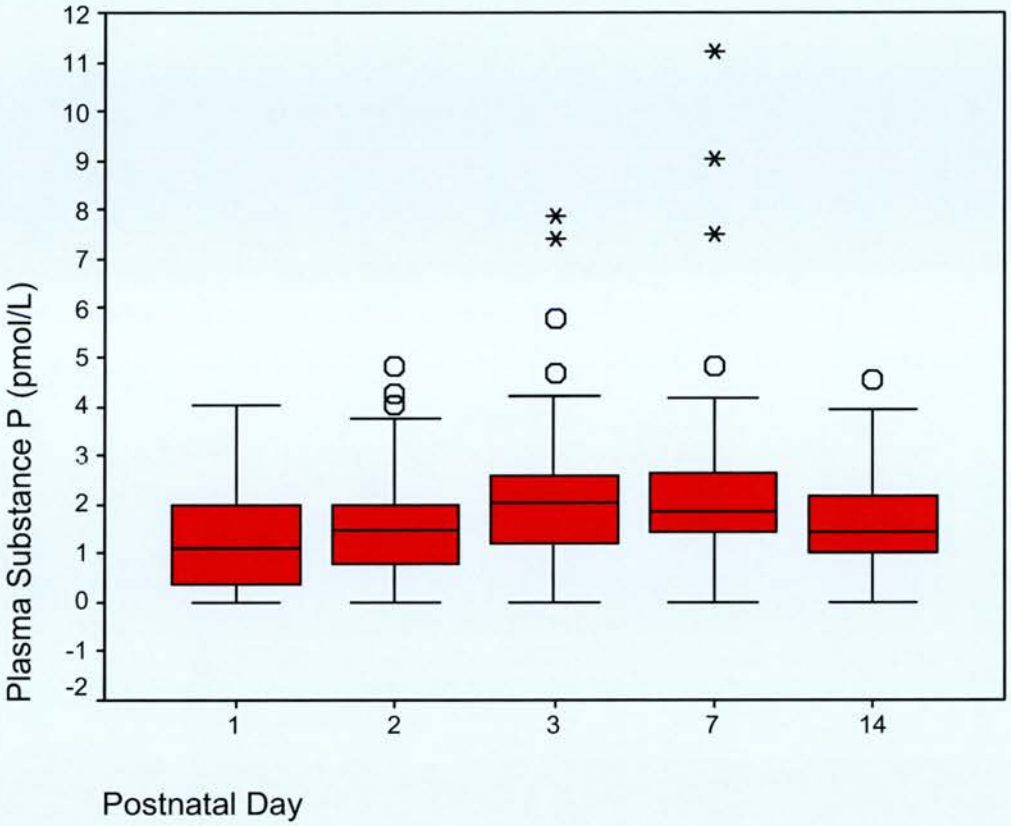



| | | | | | |
|---|---|---|---|---|---|
| Median SP (pmol/L) | 1.12 | 1.53 | 2.00 | 1.81 | 1.37 |
| N = | 70 | 76 | 142 | 115 | 82 |

O = Outlier (a value more than 1.5 box-lengths away from the box)

* = Extreme value (a value more than 3 box-lengths away from the box)

**Graph 6.3:** Variation of plasma SP with postnatal age in preterm infants ≤ 32 weeks' gestation




| | | | | | |
|---|---|---|---|---|---|
| Median SP (pmol/L) | 1.12 | 1.50 | 2.04 | 1.87 | 1.44 |
| N = | 54 | 55 | 77 | 74 | 59 |

O = Outlier (a value more than 1.5 box-lengths away from the box)

* = Extreme value (a value more than 3 box-lengths away from the box)

### 6.2.3 Perinatal factors and plasma SP

On day 1, plasma SP showed no significant differences depending on mode of delivery, presence of labour, use of antenatal steroids, maternal opiate administration, maternal epidural administration, or maternal general anaesthetic administration (Table 6.1). There was no correlation between plasma SP and cord pH ($r = -0.07$, $P = 0.61$).

**Table 6.1:** The effect of perinatal factors on plasma SP

| | | | **P value** |
|---|---|---|---|
| **Mode of delivery** | **SVD** | **CS** | |
| | *N=31* | *N=39* | |
| Median plasma SP (pmol/L) | 1.13 | 1.06 | 0.8 |
| **Presence of labour** | **Labour** | **No labour** | |
| | *N=48* | *N=22* | |
| Median plasma SP (pmol/L) | 1.16 | 1.03 | 0.8 |
| **Use of antenatal steroids** | **Yes** | **No** | |
| | *N=56* | *N=14* | |
| Median plasma SP (pmol/L) | 1.23 | 0.94 | 0.6 |
| **Maternal opiate administration** | **Yes** | **No** | |
| | *N=34* | *N=34* | |
| Median plasma SP (pmol/L) | 1.47 | 0.87 | 0.09 |
| **Maternal epidural analgesia** | **Yes** | **No** | |
| | *N=31* | *N=37* | |
| Median plasma SP (pmol/L) | 1.47 | 1.10 | 0.5 |
| **Maternal general anaesthesia** | **Yes** | **No** | |
| | *N=12* | *N=56* | |
| Median plasma SP (pmol/L) | 1.04 | 1.14 | 0.6 |

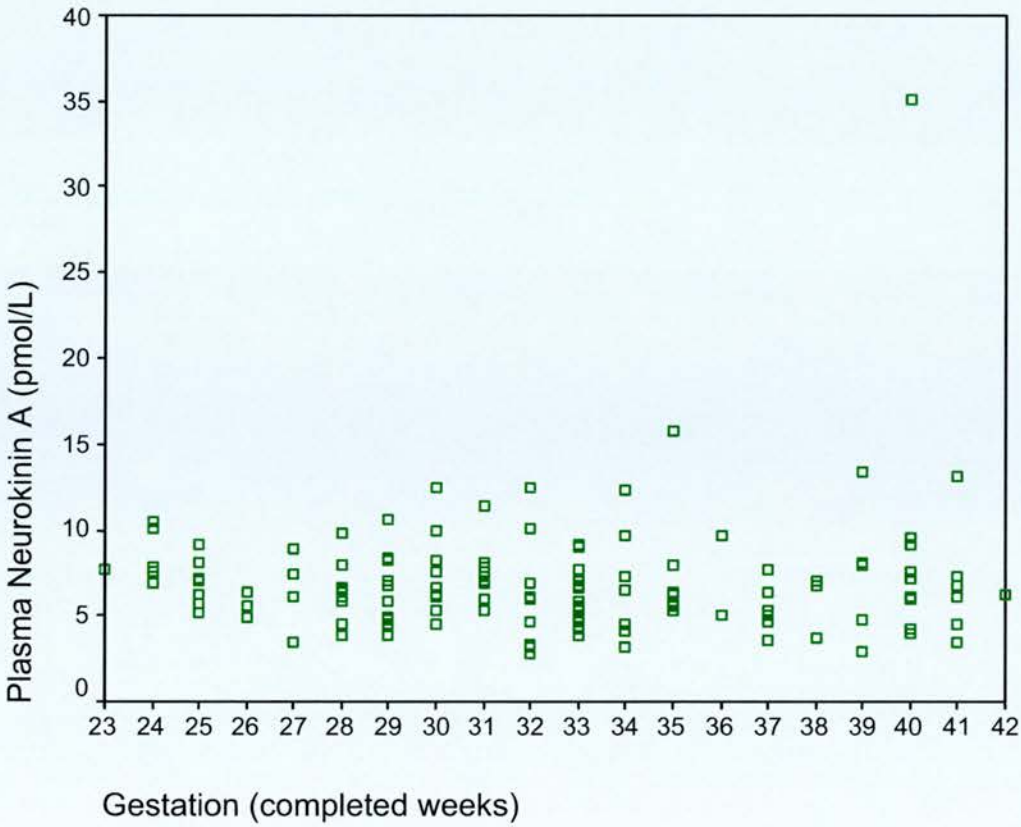
## 6.3 BASELINE CONCENTRATIONS OF PLASMA NKA IN NEONATES

Plasma NKA concentrations in neonates were higher than plasma SP. They ranged from 1.8 to 74.6 pmol/L (median 6.0 pmol/L).

### 6.3.1 Variation of plasma NKA with gestation

There was again no significant correlation between plasma NKA concentrations and gestation ($r = 0.06$, $P = 0.5$) (Graph 6.4) or birth weight ($r = 0.03$, $P = 0.7$).

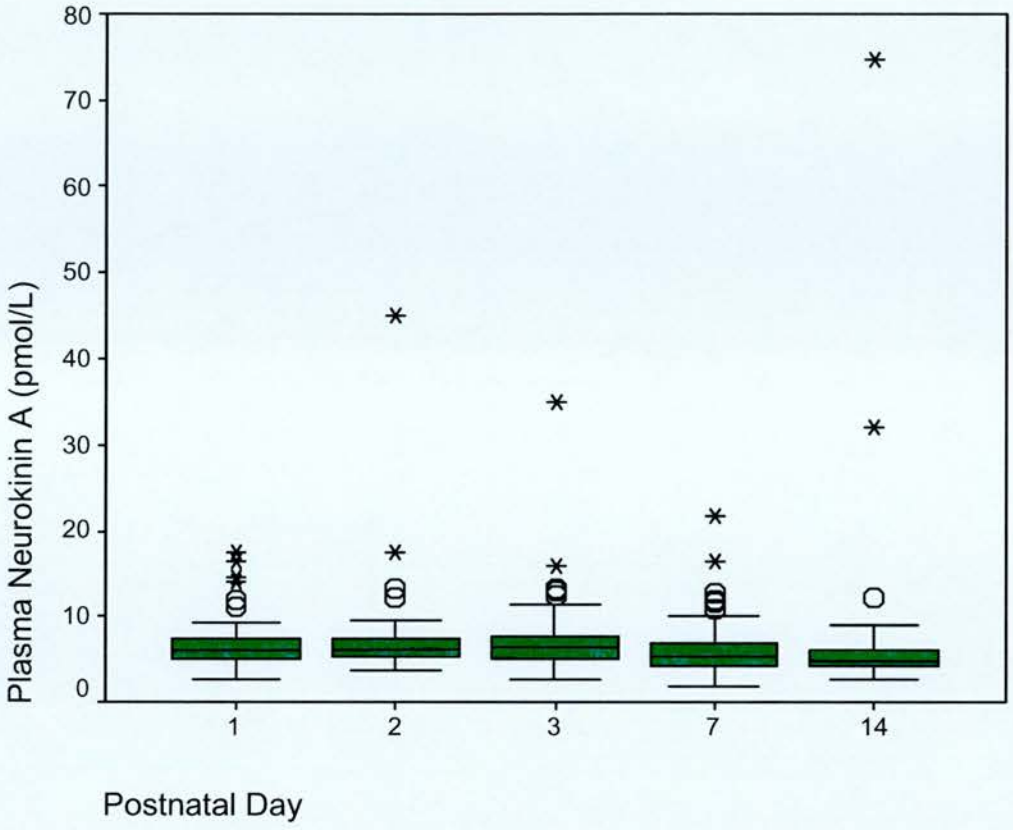
**Graph 6.4:** Variation of plasma NKA with gestation



### 6.3.2 Variation of plasma NKA with postnatal age

Postnatally, median plasma NKA showed a similar rise to SP over the first three days. Subsequently, NKA concentrations decreased by day 7 whereas SP concentrations did not decrease until day 14 (Graph 6.5). However, a one-way between subjects ANOVA showed this variation not to be significant ($F(4, 480) = 0.915$, $P = 0.6$). Although, as with SP, this pattern was more apparent in preterm infants $\leq$ 32 weeks' gestation (Graph 6.6), the ANOVA remained non-significant ($F(4, 314) = 0.283$, $P = 0.9$).

**Graph 6.5:** Variation of plasma NKA with postnatal age

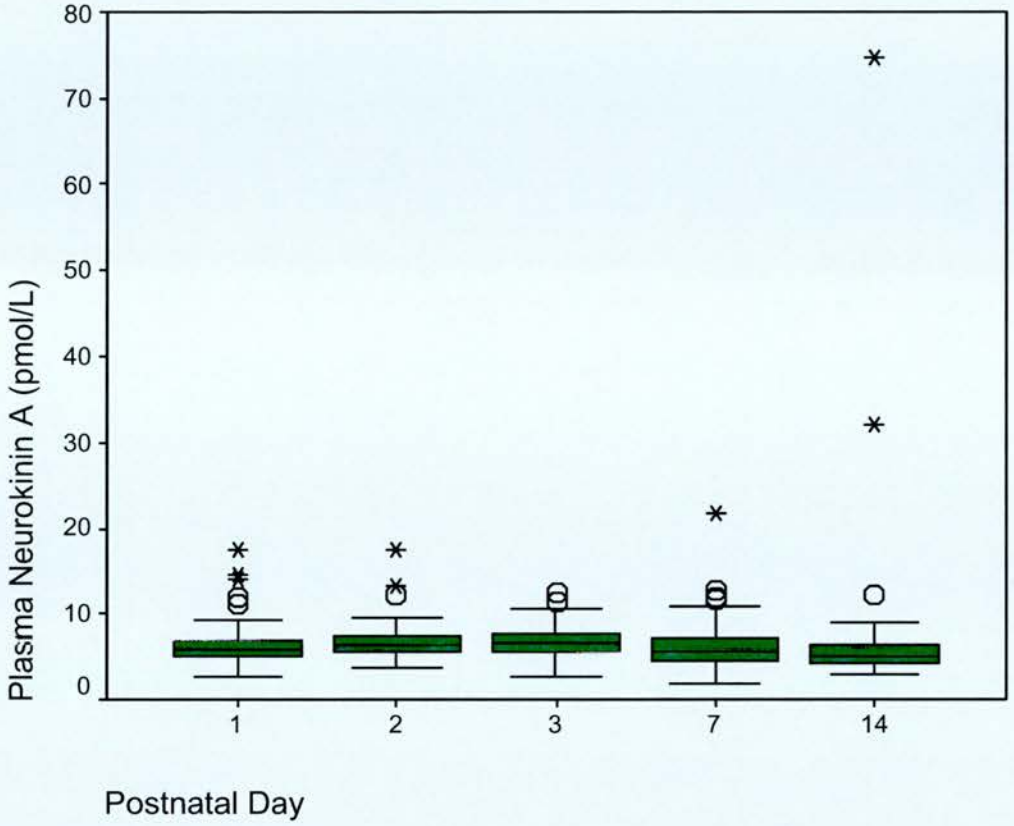



| | | | | | |
|---|---|---|---|---|---|
| Median NKA (pmol/L) | 6.15 | 6.20 | 6.46 | 5.42 | 4.72 |
| N = | 70 | 76 | 142 | 115 | 82 |

O = Outlier (a value more than 1.5 box-lengths away from the box)

* = Extreme value (a value more than 3 box-lengths away from the box)

**Graph 6.6:** Variation of plasma NKA with postnatal age in preterm infants ≤ 32 weeks' gestation




| | | | | | |
|---|---|---|---|---|---|
| Median NKA (pmol/L) | 5.83 | 6.31 | 6.62 | 5.61 | 5.04 |
| N = | 54 | 55 | 77 | 74 | 59 |

O = Outlier (a value more than 1.5 box-lengths away from the box)

* = Extreme value (a value more than 3 box-lengths away from the box)

### 6.3.3 Perinatal factors and plasma NKA

Antenatal factors which have an influence on day 1 plasma NKA concentrations were mode of delivery, the occurrence of active labour, and maternal epidural analgesia (Table 6.2). Plasma NKA concentrations were significantly higher on day 1 in infants born by caesarian section than by spontaneous vertex delivery. Concentrations were also significantly higher in the absence of labour. Maternal epidural analgesia was associated with higher concentrations of plasma NKA. Other perinatal factors did not have a significant effect on plasma NKA concentrations, although maternal opiate administration approached statistical significance. There was no correlation between plasma NKA and cord pH ($r = -0.12$, $P = 0.39$).

**Table 6.2:** The effect of perinatal factors on plasma NKA

| | | | P value |
|---|---|---|---|
| **Mode of delivery** | **SVD** | **CS** | |
| | *N=31* | *N=39* | |
| Median plasma NKA (pmol/L) | 5.60 | 6.82 | **0.003** |
| **Presence of labour** | **Labour** | **No labour** | |
| | *N=48* | *N=22* | |
| Median plasma NKA (pmol/L) | 5.82 | 6.93 | **0.005** |
| **Use of antenatal steroids** | **Yes** | **No** | |
| | *N=56* | *N=14* | |
| Median plasma NKA (pmol/L) | 6.03 | 6.29 | 0.7 |
| **Maternal opiate administration** | **Yes** | **No** | |
| | *N=34* | *N=34* | |
| Median plasma NKA (pmol/L) | 5.69 | 6.93 | 0.06 |
| **Maternal epidural analgesia** | **Yes** | **No** | |
| | *N=31* | *N=37* | |
| Median plasma NKA (pmol/L) | 6.94 | 5.64 | **0.008** |
| **Maternal general anaesthesia** | **Yes** | **No** | |
| | *N=12* | *N=56* | |
| Median plasma NKA (pmol/L) | 6.74 | 6.12 | 0.4 |

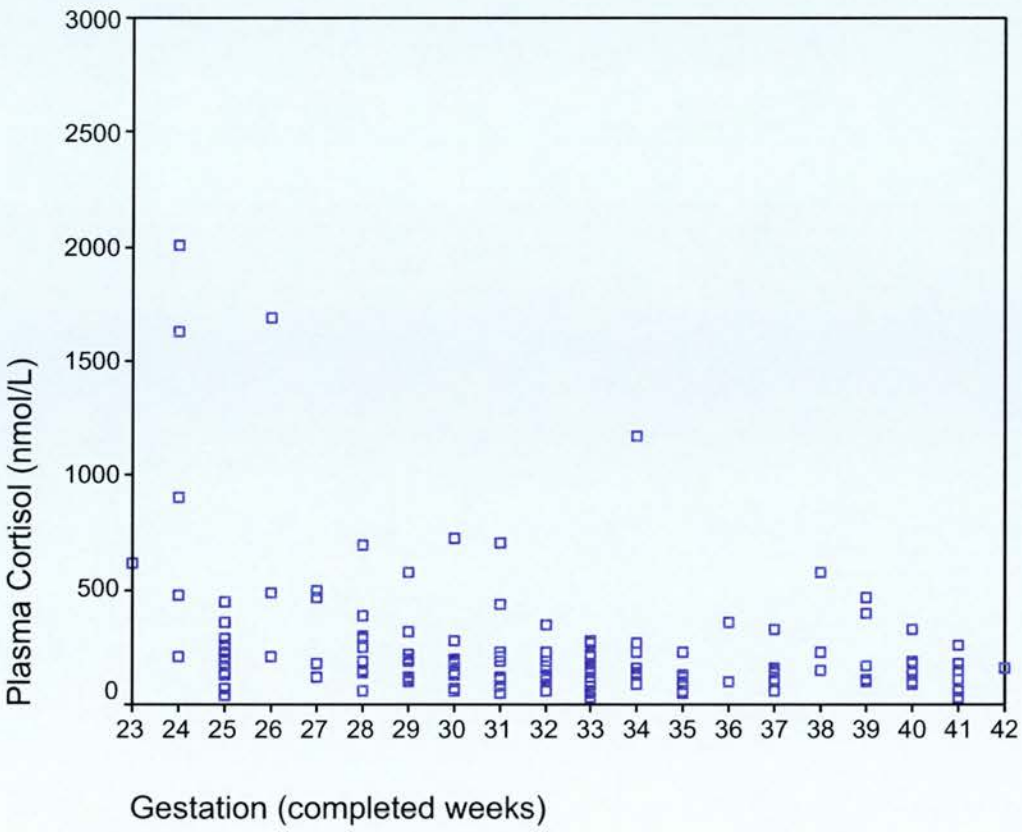
## 6.4 BASELINE CONCENTRATIONS OF PLASMA CORTISOL IN NEONATES

Plasma cortisol concentrations in this study ranged from 21.4 to 2842.5 nmol/L (median 171.2 nmol/L).

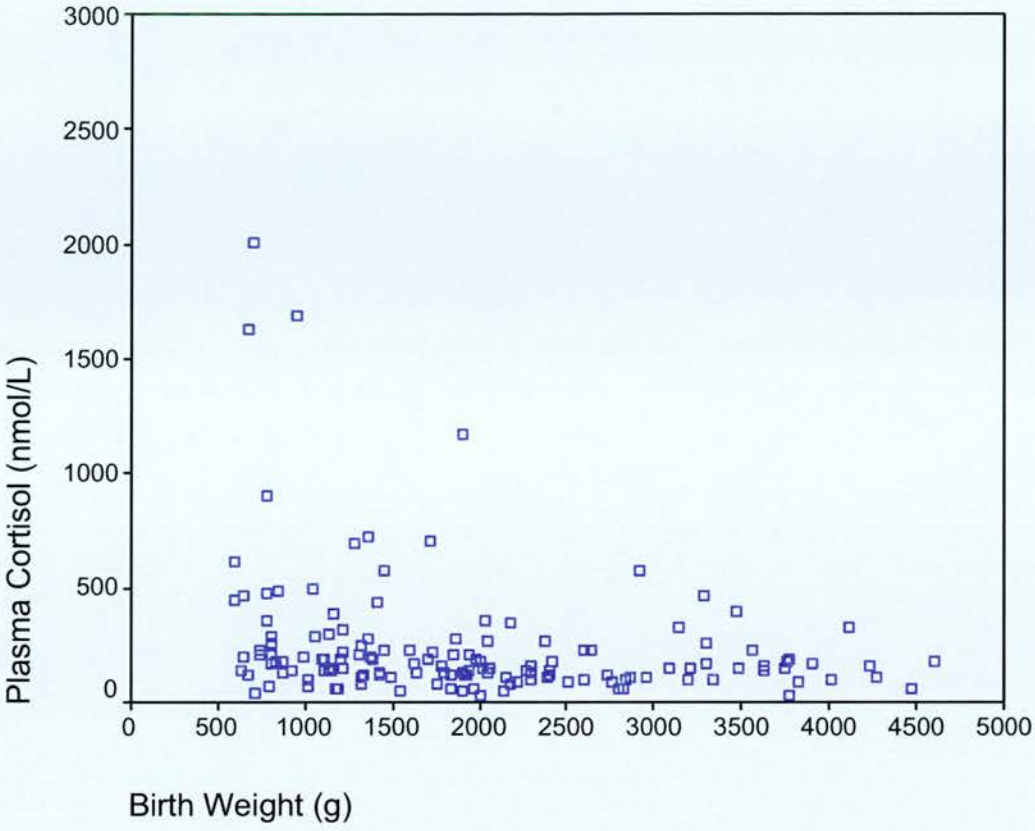
### 6.4.1 Variation of plasma cortisol with gestation

Day 1 plasma cortisol showed significant negative correlations with gestation ($r = -0.32$, $P < 0.001$) (Graph 6.7) and birth weight ($r = -0.26$, $P = 0.002$) (Graph 6.8).

**Graph 6.7:** Variation of plasma cortisol with gestation



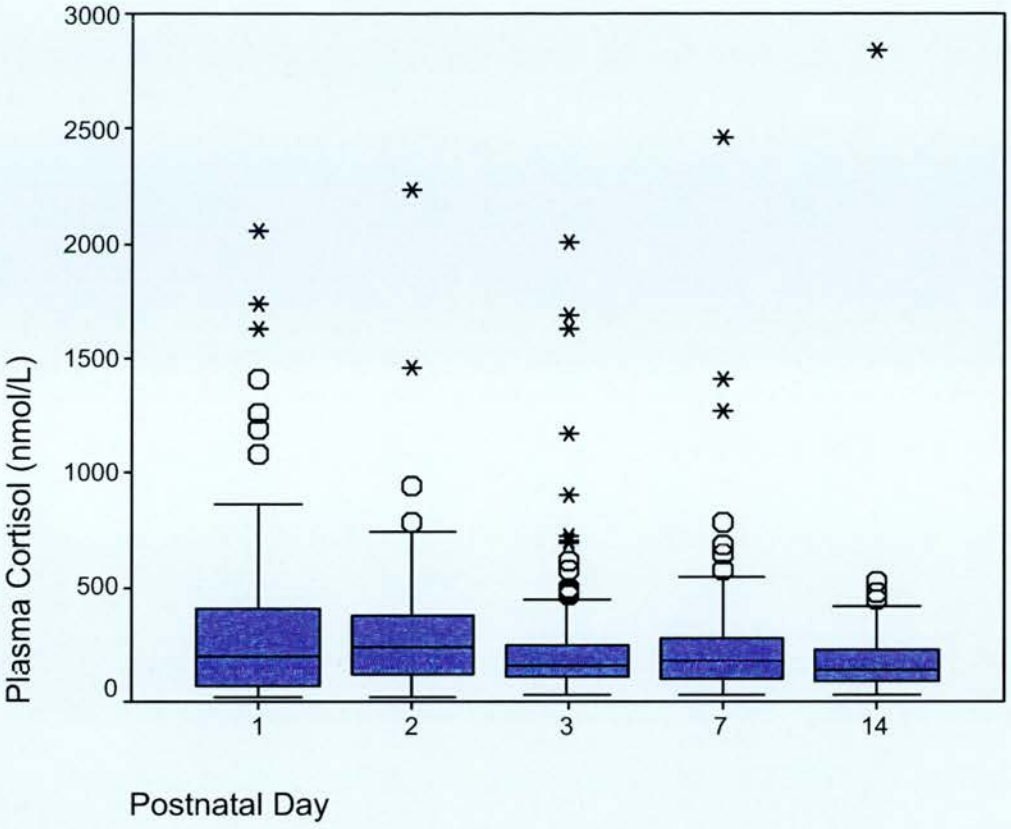
**Graph 6.8:** Variation of plasma cortisol with birth weight



### 6.4.2 Variation of plasma cortisol with postnatal age

Postnatally, plasma cortisol concentrations on days 1 & 2 were higher than days 3, 7 and 14 (Graph 6.9). As the plasma cortisol data were highly skewed, and the Levene test for homogeneity-of-variance remained highly significant ($P < 0.001$) even with $\log_{10}$ plasma cortisol values, a non-parametric test was used. The Kruskal-Wallis test showed the postnatal variation to be non-significant ($\chi^2$ (4) = 8.83, P = 0.065).

**Graph 6.9:** Variation of plasma cortisol with postnatal age




| | | | | | |
|---|---|---|---|---|---|
| Median cortisol (nmol/L) | 198.2 | 234.4 | 159.2 | 177.3 | 143.7 |
| N = | 70 | 75 | 142 | 114 | 82 |

O = Outlier (a value more than 1.5 box-lengths away from the box)

* = Extreme value (a value more than 3 box-lengths away from the box)

### 6.4.3 Perinatal factors and plasma cortisol

Antenatal factors which appeared to have an effect on day 1 plasma cortisol concentrations, but did not reach statistical significance, were mode of delivery and labour (Table 6.3). There was a trend towards higher cortisol concentrations with spontaneous vertex deliveries rather than caesarian section, and with labour. There was no significant effect of antenatal steroids on day 1 plasma cortisol concentrations. There was no correlation between plasma cortisol and cord pH ($r = 0.013$, $P = 0.92$).

**Table 6.3:** The effect of perinatal factors on plasma cortisol

| | | | P value |
|---|---|---|---|
| **Mode of delivery** | **SVD** | **CS** | |
| | *N=31* | *N=39* | |
| Median plasma cortisol (nmol/L) | 275.4 | 135.5 | 0.06 |
| **Presence of labour** | **Labour** | **No labour** | |
| | *N=48* | *N=22* | |
| Median plasma cortisol (nmol/L) | 251.0 | 131.5 | 0.06 |
| **Use of antenatal steroids** | **Yes** | **No** | |
| | *N=56* | *N=14* | |
| Median plasma cortisol (nmol/L) | 185.2 | 210.3 | 0.5 |
| **Maternal opiate administration** | **Yes** | **No** | |
| | *N=34* | *N=34* | |
| Median plasma cortisol (nmol/L) | 216.5 | 185.2 | 0.5 |
| **Maternal epidural analgesia** | **Yes** | **No** | |
| | *N=31* | *N=37* | |
| Median plasma cortisol (nmol/L) | 154.3 | 210.2 | 0.2 |
| **Maternal general anaesthesia** | **Yes** | **No** | |
| | *N=12* | *N=56* | |
| Median plasma cortisol (nmol/L) | 172.9 | 193.4 | 0.7 |

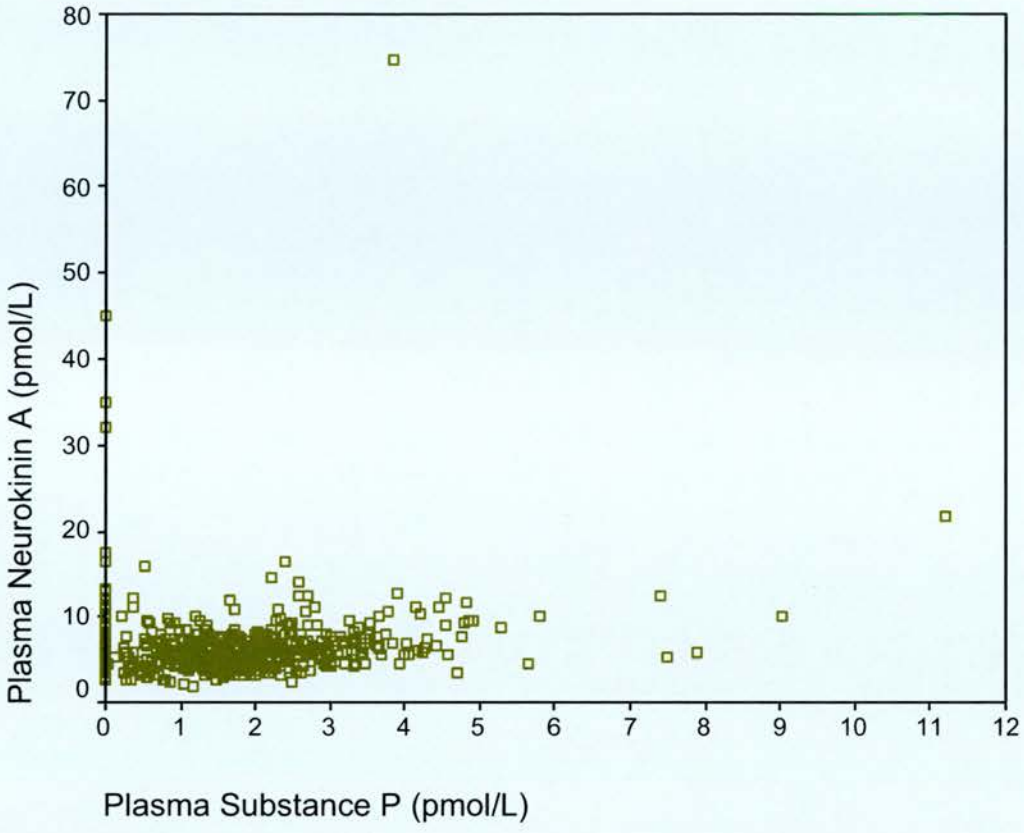
## 6.5 CORRELATION BETWEEN PLASMA HORMONES

There was some correlation between plasma SP and NKA, but no significant correlation between either neuropeptide and plasma cortisol (Table 6.4 and Graph 6.10).

**Table 6.4:** Correlations between plasma hormones

| | N | r | P value |
|---|---|---|---|
| SP and NKA | 551 | 0.13 | **0.003** |
| SP and cortisol | 549 | 0.06 | 0.14 |
| NKA and cortisol | 549 | 0.03 | 0.52 |

**Graph 6.10:** Scatterplot of paired plasma NKA and plasma SP concentrations




## 6.6 DISCUSSION

Of significant importance was that the plasma SP concentrations found in neonates in this rigorously conducted study were lower than those published elsewhere. Scholle *et al* reported mean (SE) plasma SP concentrations of 20.2 (4.6) pmol/L in 32 preterm infants, and 28.4 (5.7) pmol/L in 19 term infants[190]. However, the results in Scholle's study were from unextracted plasma samples, which have been shown to yield higher concentrations of SP due to cross-reactivity (Section 2.1). There was also no description of sample handling and preservation procedures, which has been shown to be of importance. Furthermore, despite claiming that their study was 'designed to determine the plasma concentration pattern of [SP] during the first year

of life in full-term and preterm infants', Scholle *et al* only studied a small population of infants, and the investigations were performed any time after the third week of life, with results plotted according to the infants' corrected gestational age (which ranged from –4 to 63 weeks (0 being term)). This gave rise to a very heterogeneous group of infants, with a small number of infants having different numbers of plasma samples contributing to the final results.

11% of the plasma SP results from the current study yielded a value of 0.0 pmol/L, but these were probably genuinely undetectable concentrations as NKA remained detectable in all the same samples, with concentrations ranging from 2.6 to 45.0 pmol/L (median 6.6 pmol/L). Moreover, other investigators have also reported undetectable concentrations of plasma SP, not only in infants[190], but also in adults[192;196;197]. In addition, we now know that unextracted plasma gives higher concentration measurements than extracted samples, and hence the accuracy of other published reports on high plasma SP concentrations (300-500 pmol/L) where sample extraction had not been performed[138;198;199], is open to question.

Plasma NKA concentrations were found to be higher than those of plasma SP, and no sample yielded an undetectable result. This is the first study of plasma NKA in newborn infants, hence no comparisons are available. Most of the *in vivo* research into NKA and pain has been in synovial fluid rather than plasma[200-203].

When looking at day 1 samples from each infant, no correlation was found between plasma neuropeptide concentrations, and gestation or birth weight (Sections 6.2.1

and 6.3.1). However, day 1 samples can also be a reflection of perinatal circumstances, hence the effect of various perinatal factors of the neuropeptides was examined. Plasma SP concentrations were not affected by perinatal factors (Section 6.2.3) but unexpectedly, plasma NKA concentrations were *higher* in the absence of labour, in infants born by caesarian section, and if maternal epidural analgesia was administered (but not general anaesthesia) (Section 6.3.3). This was contrary to expectation, as NKA concentrations would be predicted to rise with pain. Nevertheless, although labour is painful to women, it is not known to cause pain to the infant. Also, a normal delivery may be *uncomfortable* to the infant, but is not known to be *painful*. It is recognised that labour induces endogenous β-endorphin production in women[204;205], as well as fetal β-endorphin levels as determined from cord blood after delivery[206;207]. The fetal β-endophins may act as a natural analgesic to the infant. Epidural analgesia, however, would reduce the pain of labour to the mother. However, although epidural analgesia has been shown to attenuate maternal β-endorphin production[208], it does not affect fetal β-endorphin levels[204;205]. Maternal opiate administration was associated with a trend towards lower neonatal plasma NKA concentrations, which would be as anticipated.

With postnatal age, both plasma SP and NKA showed a gradual rise over the first three days, followed by a subsequent decline, but was only statistically significant for SP. This pattern was more marked in preterm infants less than 32 weeks' gestation. Infants receiving intensive care are more likely to undergo multiple painful or stressful procedures during the first days of initial stabilisation, which could explain

this finding. Also, the more premature the infant, the more invasive procedures required for stabilisation.

Plasma cortisol concentrations in this study were similar to those found by Midgley *et al*[209], and fell with increasing gestation and postnatal age, again echoing findings by other investigators[209;210]. There was no significant effect of antenatal steroids on day 1 plasma cortisol, a finding contrary to previous data obtained by some researchers[211]. However, other investigators have found no effect of antenatal steroids on cord cortisol concentrations[212]. Also, the time between antenatal steroid administration and delivery was not recorded in the current study, and this has been shown to affect cord cortisol concentrations[213].

Finally, the current study shows that a weak correlation exists between plasma SP and NKA, but not between either neuropeptide and cortisol. The correlation between the neuropeptides is unlikely to be due to cross-reactivity in the radioimmunoassays, as this is <2%. It is more likely to be due to the fact that the same gene codes for both neuropeptides (PPT-A gene, Section 1.5.2.1) and hence upregulation of the gene for one peptide could result in increased production of the other peptide. NKA is co-synthesised and co-secreted with SP, but has a different regional distribution and asserts its effects via a different receptor to result in different biological functions.

The lack of correlation between the neuropeptides and cortisol serves to reinforce the theory that while cortisol may be a good marker for non-painful stress, the

neuropeptides may be better markers for pain. This hypothesis is explored in the next chapter.

# 7. CLINICAL STUDY: THE EFFECTS OF PERSISTENT PAIN AND ASSISTED VENTILATION ON PLASMA SUBSTANCE P AND NEUROKININ A IN NEONATES

## 7.1 INTRODUCTION AND AIMS

Once the demographics of SP and NKA had been established in neonates, it was possible to proceed with investigations into the effect of persistent pain and ventilation on the concentrations of SP and NKA.

The specific aims here were:

i) To observe how conditions which are likely to result in persistent pain, e.g. meningitis, intraventricular haemorrhage (grades III and IV), necrotising enterocolitis (NEC) and surgery, affect SP and NKA concentrations, and whether analgesia makes a difference.

ii) To determine whether assisted ventilation is a cause of persistent pain or non-painful stress to otherwise healthy but premature neonates. At the time of this study, it was possible to capitalise on the Edinburgh Neonatal Unit's involvement in the international NEOPAIN study (Chapter 5).

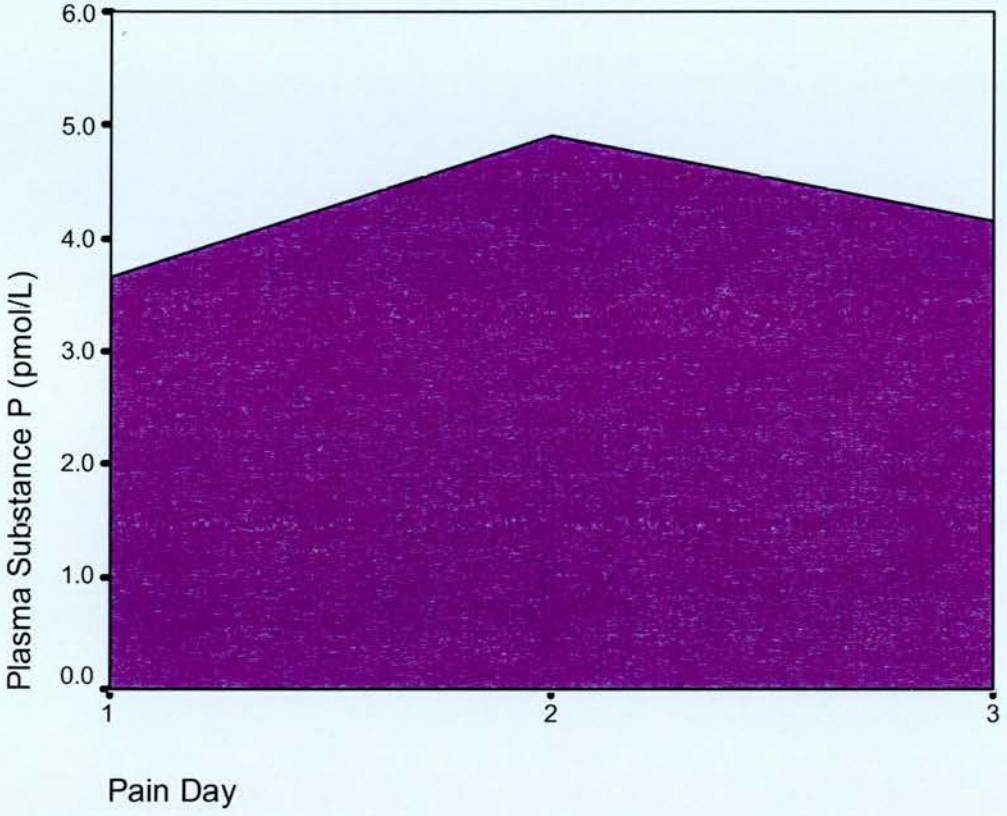
iii) Comparison was made between Premature Infant Pain Profile (PIPP) scores and physiological markers of pain and distress (heart rate and its variability) in ventilated versus non-ventilated infants. Also, any correlation between these markers and SP or NKA concentrations was investigated.

The methods used are described in Chapter 5. Infants in pain were excluded from analyses for variation with ventilation and analgesia (Sections 7.2 to 7.3).

## 7.2 VARIATION WITH PERSISTENT PAIN

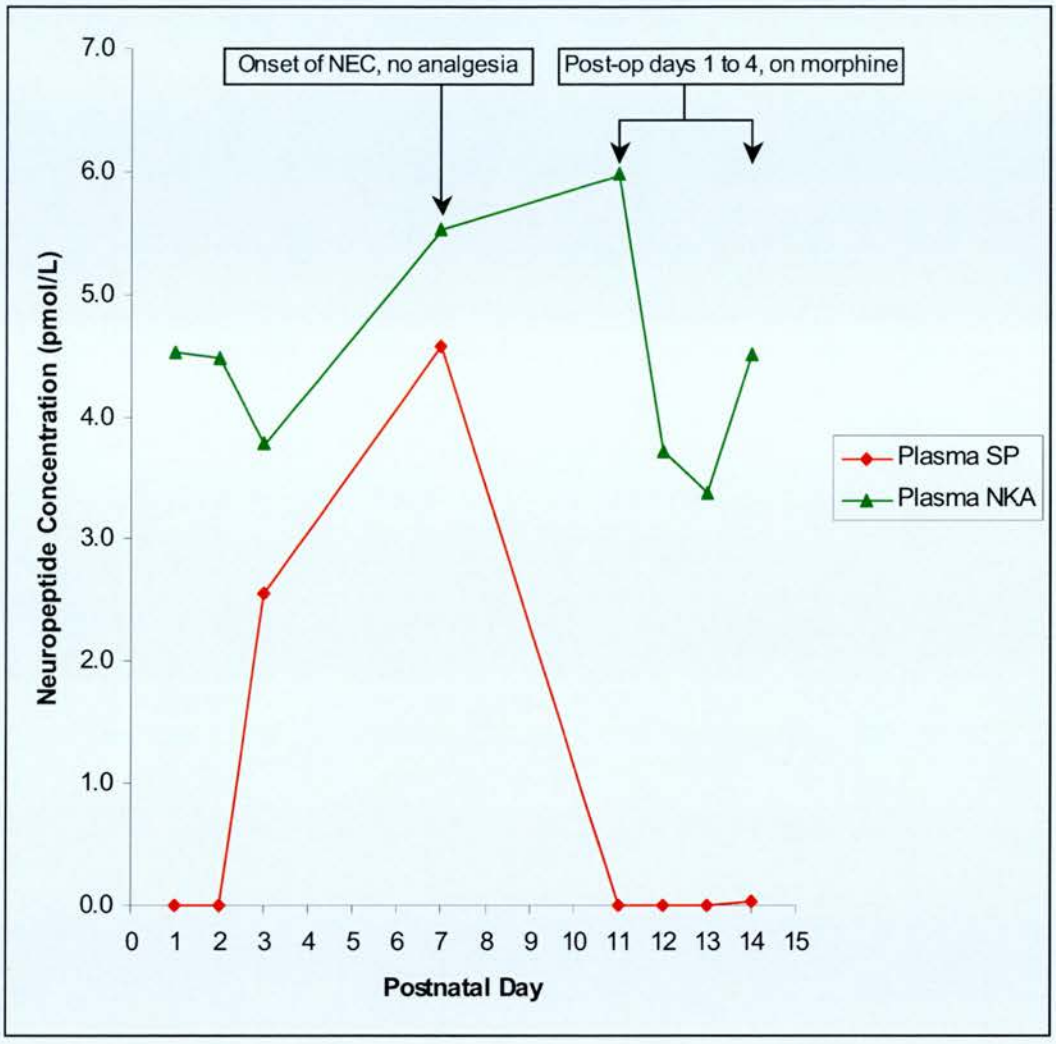
As described in Section 5.3.1.2, 19 neonates were believed to be suffering persistent pain. Area under the curve (AUC) was calculated for three consecutive daily neuropeptide concentrations during the pain duration (Graph 7.1). Comparing the pain group with their controls, there was no significant difference in median AUC for either plasma SP (pain = 3.80, control = 3.03, P = 0.82) or plasma NKA (pain = 10.2, control = 12.3, P = 0.33). $Log_{10}$ of plasma cortisol values was computed and AUC for the log values calculated. There was no significant difference in mean AUC for log-transformed plasma cortisol (pain = 4.68, control = 4.45, P = 0.25).

**Graph 7.1:** Example of AUC estimation for three daily consecutive neuropeptide concentrations



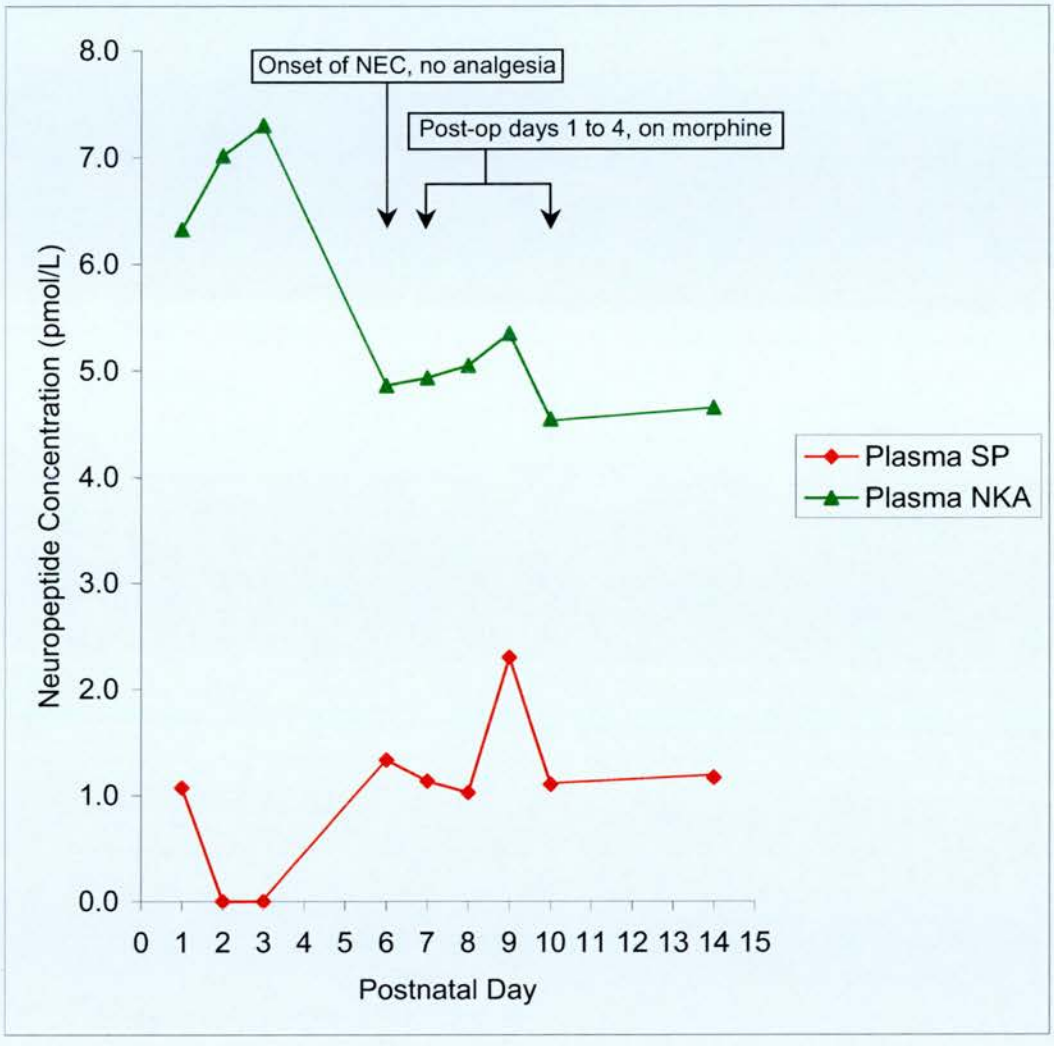

Longitudinal graphs of plasma peptide concentrations over time were also examined for individual infants and no consistent change noted with pain or analgesia administration (Graphs 7.2a and 7.2b). Only 2 infants had plasma samples taken before and after the painful event and the use of analgesia. The rest either did not have samples taken before the onset of the painful event, or the infant was already on analgesia by the time of enrolment.

**Graph 7.2a:** Example of an individual infant's plasma neuropeptide concentrations




over time (Study subject 97)

**Graph 7.2b:** Example of an individual infant's plasma neuropeptide concentrations over time (Study subject 102)



## 7.3 VARIATION WITH ASSISTED VENTILATION

Median plasma neuropeptide values (pmol/L) and plasma cortisol values (nmol/L) are tabulated. Table 7.1 shows SP results from all neonates grouped by ventilation and stratified by postnatal day. Tables 7.2 and 7.3 show NKA and cortisol results in a similar way. Tables 7.1a, 7.2a and 7.3a show the corresponding results for infants ≤ 32 weeks' gestation.

**Table 7.1:** The effect of assisted ventilation on median plasma substance P concentrations, stratified by postnatal day

| Postnatal day | Ventilated | | Not ventilated | | P value |
|---|---|---|---|---|---|
| | N | SP (pmol/L) | N | SP (pmol/L) | |
| 1 | 39 | 1.10 | 31 | 1.47 | 0.7 |
| 2 | 40 | 1.63 | 36 | 1.34 | 0.6 |
| 3 | 46 | 2.03 | 96 | 2.00 | 0.2 |
| 7 | 30 | 1.89 | 85 | 1.75 | 0.4 |
| 14 | 20 | 1.51 | 62 | 1.33 | 0.5 |

**Table 7.1a:** The effect of assisted ventilation on median plasma substance P concentrations, stratified by postnatal day **(infants ≤ 32 weeks' gestation only)**

| Postnatal day | Ventilated | | Not ventilated | | P value |
|---|---|---|---|---|---|
| | N | SP (pmol/L) | N | SP (pmol/L) | |
| 1 | 36 | 1.09 | 18 | 1.47 | 0.6 |
| 2 | 37 | 1.55 | 18 | 1.31 | 0.5 |
| 3 | 38 | 1.71 | 39 | 2.06 | 1.0 |
| 7 | 28 | 1.95 | 46 | 1.80 | 0.4 |
| 14 | 20 | 1.51 | 39 | 1.42 | 0.6 |

**Table 7.2:** The effect of assisted ventilation on median plasma neurokinin A concentrations, stratified by postnatal day

| Postnatal day | Ventilated | | Not ventilated | | P value |
|---|---|---|---|---|---|
| | N | NKA (pmol/L) | N | NKA (pmol/L) | |
| 1 | 39 | 5.64 | 31 | 6.82 | **0.01** |
| 2 | 40 | 5.85 | 36 | 7.06 | **0.01** |
| 3 | 46 | 6.20 | 96 | 6.67 | 0.2 |
| 7 | 30 | 6.12 | 85 | 5.26 | **0.02** |
| 14 | 20 | 5.40 | 62 | 4.64 | **0.01** |

**Table 7.2a:** The effect of assisted ventilation on median plasma neurokinin A concentrations, stratified by postnatal day **(infants ≤ 32 weeks' gestation only)**

| Postnatal day | Ventilated | | Not ventilated | | P value |
|---|---|---|---|---|---|
| | N | NKA (pmol/L) | N | NKA (pmol/L) | |
| 1 | 36 | 5.58 | 18 | 6.16 | 0.1 |
| 2 | 37 | 5.79 | 18 | 7.39 | **0.01** |
| 3 | 38 | 6.39 | 39 | 6.89 | 0.2 |
| 7 | 28 | 6.12 | 46 | 5.17 | **0.05** |
| 14 | 20 | 5.40 | 39 | 4.81 | 0.06 |

**Table 7.3:** The effect of assisted ventilation on median plasma cortisol concentrations, stratified by postnatal day

| Postnatal day | Ventilated | | Not ventilated | | P value |
|---|---|---|---|---|---|
| | N | Cortisol (nmol/L) | N | Cortisol (nmol/L) | |
| 1 | 39 | 319.8 | 31 | 85.4 | **< 0.001** |
| 2 | 40 | 325.7 | 35 | 128.1 | **< 0.001** |
| 3 | 46 | 209.1 | 96 | 139.5 | **< 0.001** |
| 7 | 30 | 260.0 | 84 | 167.1 | **0.001** |
| 14 | 20 | 211.6 | 62 | 125.9 | **0.002** |

**Table 7.3a:** The effect of assisted ventilation on median plasma cortisol concentrations, stratified by postnatal day **(infants ≤ 32 weeks' gestation only)**

| Postnatal day | Ventilated | | Not ventilated | | P value |
|---|---|---|---|---|---|
| | N | Cortisol (nmol/L) | N | Cortisol (nmol/L) | |
| 1 | 34 | 309.7 | 18 | 99.5 | **0.006** |
| 2 | 35 | 310.0 | 17 | 107.8 | **0.001** |
| 3 | 36 | 209.1 | 39 | 133.3 | **0.002** |
| 7 | 26 | 273.1 | 45 | 176.9 | **0.009** |
| 14 | 19 | 211.6 | 38 | 147.7 | **0.05** |

Plasma SP concentrations were not found to be significantly different in ventilated infants. Plasma NKA concentrations showed an unexpected variation, being significantly lower in ventilated infants on days 1 & 2, and higher on days 7 & 14. Plasma cortisol concentrations were very significantly higher in ventilated infants across all postnatal days, as would be expected of a marker of stress, whether painful or not.

## 7.4 Variation with analgesia in ventilated infants (the NEOPAIN subgroup)

In the subgroup of ventilated infants ≤ 32 weeks' gestation who were also enrolled into the NEOPAIN study, 21 received morphine therapy and 13 received placebo. AUC was calculated as before for neuropeptide and $\log_{10}$ cortisol concentrations on postnatal days 1 to 3 (Table 7.4). Comparing infants on morphine therapy with those on placebo therapy, there were no significant differences in median AUC for plasma SP or mean AUC for log-transformed plasma cortisol. Median AUC for plasma NKA was significantly lower in those ventilated infants who received morphine.

**Table 7.4:** Median AUC for neuropeptide and $log_{10}$ cortisol concentrations in ventilated infants on postnatal days 1 to 3, morphine versus placebo groups

| | **Median AUC** | | **P value** |
|---|---|---|---|
| | Morphine | Placebo | |
| | *N=19* | *N=12* | |
| Substance P (pmol/L) | 3.21 | 3.38 | 0.5 |
| Neurokinin A (pmol/L) | 11.4 | 13.0 | **0.05** |
| $Log_{10}$ cortisol (nmol/L) | 4.94 | 5.07 | 0.6 |

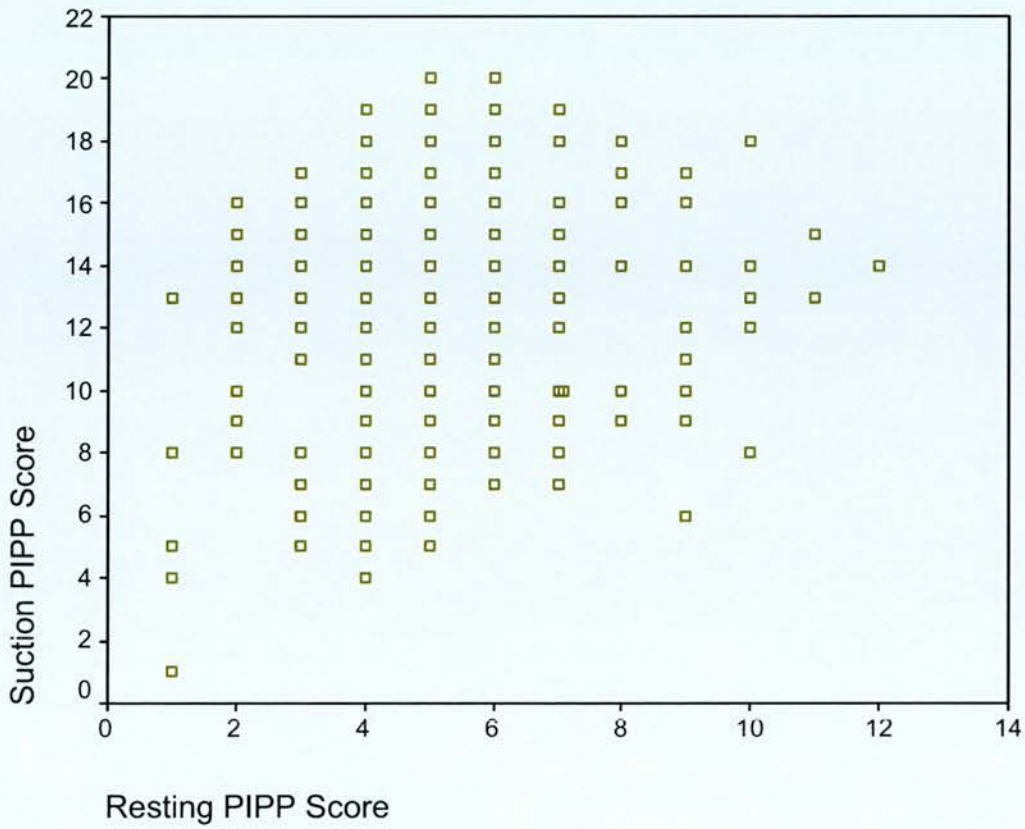
## 7.5 CORRELATION WITH PIPP SCORES AND VARIATION OF PIPP SCORES WITH ASSISTED VENTILATION

Neither plasma SP nor NKA showed an appreciable correlation with either the resting or suction PIPP scores, but plasma NKA showed a positive correlation with PIPP scores from a pain stimulus (Table 7.5). Plasma cortisol showed a negative correlation with suction PIPP scores and pain stimulus PIPP scores. There was, as expected, a positive correlation between resting and suction PIPP scores ($r = 0.21$, $P = 0.001$), and suction and pain stimulus PIPP scores ($r = 0.22$, $P = 0.01$) (Graphs 7.3 and 7.4).

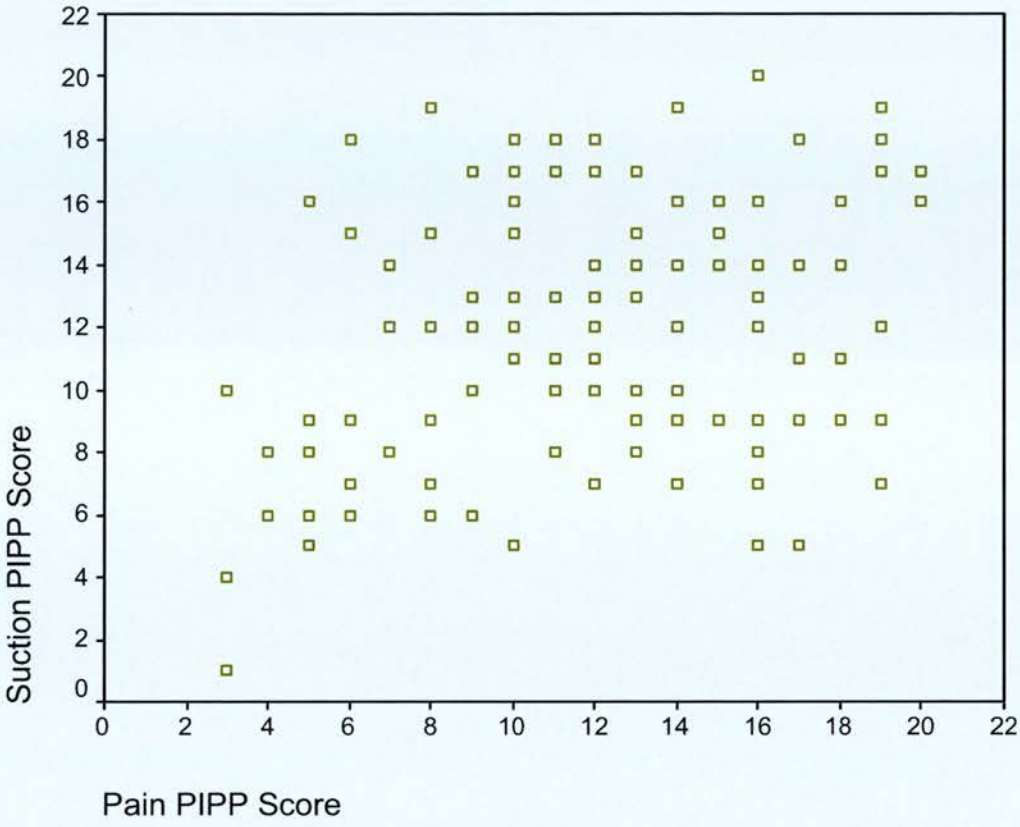
**Table 7.5:** Correlations between hormones and PIPP scores

| | **Resting PIPP score r** | **P value** | **Suction PIPP score r** | **P value** | **Pain stimulus PIPP score r** | **P value** |
|---|---|---|---|---|---|---|
| SP | 0.05 | 0.5 | 0.04 | 0.6 | -0.09 | 0.3 |
| NKA | 0.02 | 0.8 | -0.08 | 0.2 | 0.32 | **< 0.001** |
| Cortisol | 0.05 | 0.4 | -0.22 | **< 0.001** | -0.24 | **0.01** |

**Graph 7.3:** Scatterplot of paired suction and resting PIPP scores



**Graph 7.4:** Scatterplot of paired suction and pain stimulus PIPP scores




It was noted that resting PIPP scores tended to be higher in infants receiving assisted ventilation than non-ventilated infants (Table 7.6). Although the difference in scores between ventilated and non-ventilated infants was statistically significant on postnatal days 1 and 2, the actual difference of 1 point in the score was clinically insignificant. With PIPP scores in response to suction, the reverse was found, though not statistically significant (Table 7.7). Unfortunately the number of ventilated infants who had a PIPP score performed with a painful stimulus was very small, as most of these infants had arterial lines from which blood was sampled, thereby avoiding venepuncture (Table 7.8).

**Table 7.6:** The effect of assisted ventilation on median resting PIPP scores, stratified by postnatal day

| **Postnatal day** | **Ventilated** | | **Not ventilated** | | **P value** |
|---|---|---|---|---|---|
| | **N** | **Median resting PIPP score** | **N** | **Median resting PIPP score** | |
| 1 | 35 | 5 | 27 | 4 | **0.02** |
| 2 | 32 | 5 | 24 | 4 | **0.003** |
| 3 | 28 | 6 | 22 | 4.5 | 0.07 |
| 7 | 20 | 5 | 20 | 5 | 0.9 |
| 14 | 12 | 6 | 14 | 5 | 0.6 |

**Table 7.7:** The effect of assisted ventilation on median suction PIPP scores, stratified by postnatal day

| Postnatal day | Ventilated | | Not ventilated | | P value |
|---|---|---|---|---|---|
| | N | Median suction PIPP score | N | Median suction PIPP score | |
| 1 | 35 | 11 | 27 | 13 | 0.3 |
| 2 | 32 | 10 | 24 | 12 | 0.2 |
| 3 | 28 | 11 | 22 | 13.5 | 0.3 |
| 7 | 20 | 11 | 20 | 12.5 | 0.4 |
| 14 | 12 | 11.5 | 14 | 12 | 0.7 |

**Table 7.8:** The effect of assisted ventilation on median pain stimulus PIPP scores, stratified by postnatal day

| **Postnatal day** | **Ventilated** | | **Not ventilated** | | **P value** |
|---|---|---|---|---|---|
| | **N** | **Median pain PIPP score** | **N** | **Median pain PIPP score** | |
| 1 | 2 | 15.5 | 17 | 15 | 0.6 |
| 2 | 2 | 10.5 | 19 | 15 | 0.2 |
| 3 | 3 | 10 | 19 | 12 | 0.08 |
| 7 | 6 | 11.5 | 19 | 11 | 0.6 |
| 14 | 11 | 14 | 11 | 10 | 0.3 |

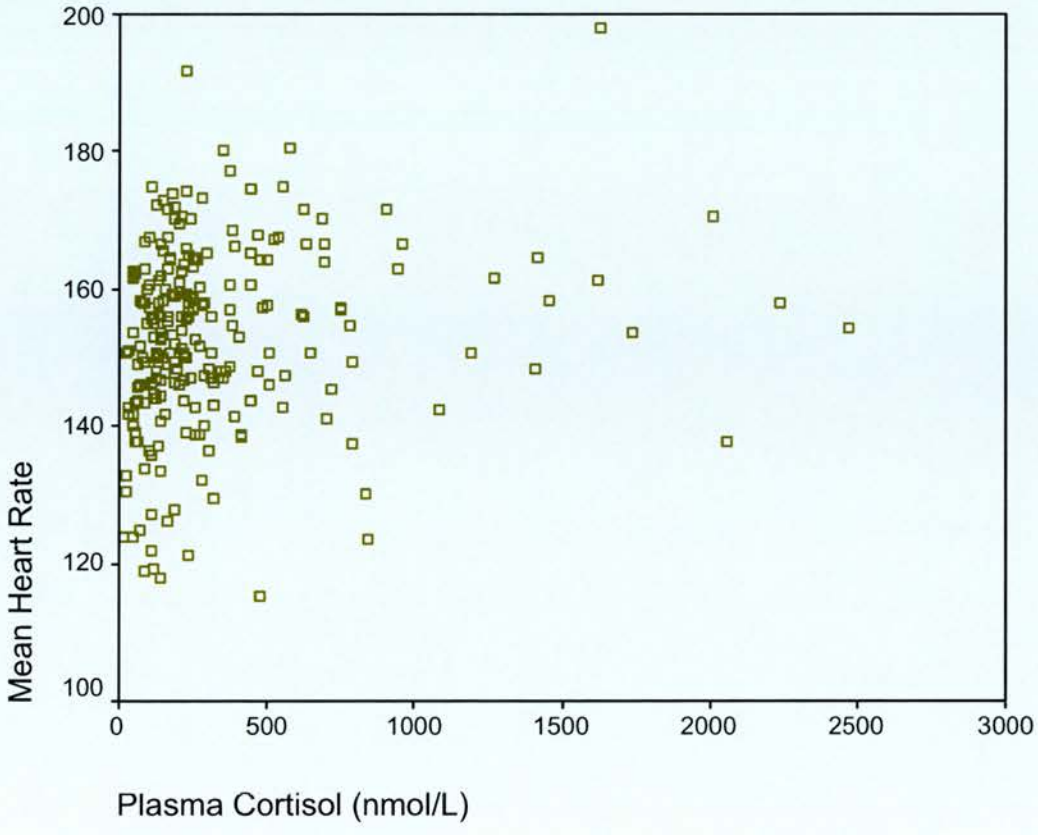
## 7.6 CORRELATION WITH HEART RATE AND HEART RATE VARIABILITY, AND VARIATION OF HEART RATE DATA WITH ASSISTED VENTILATION

Plasma SP and NKA showed no correlation with either mean heart rate or heart rate variability (Table 7.9). Plasma cortisol, however, correlated positively with mean heart rate and negatively with heart rate variability (Graphs 7.5 and 7.6).

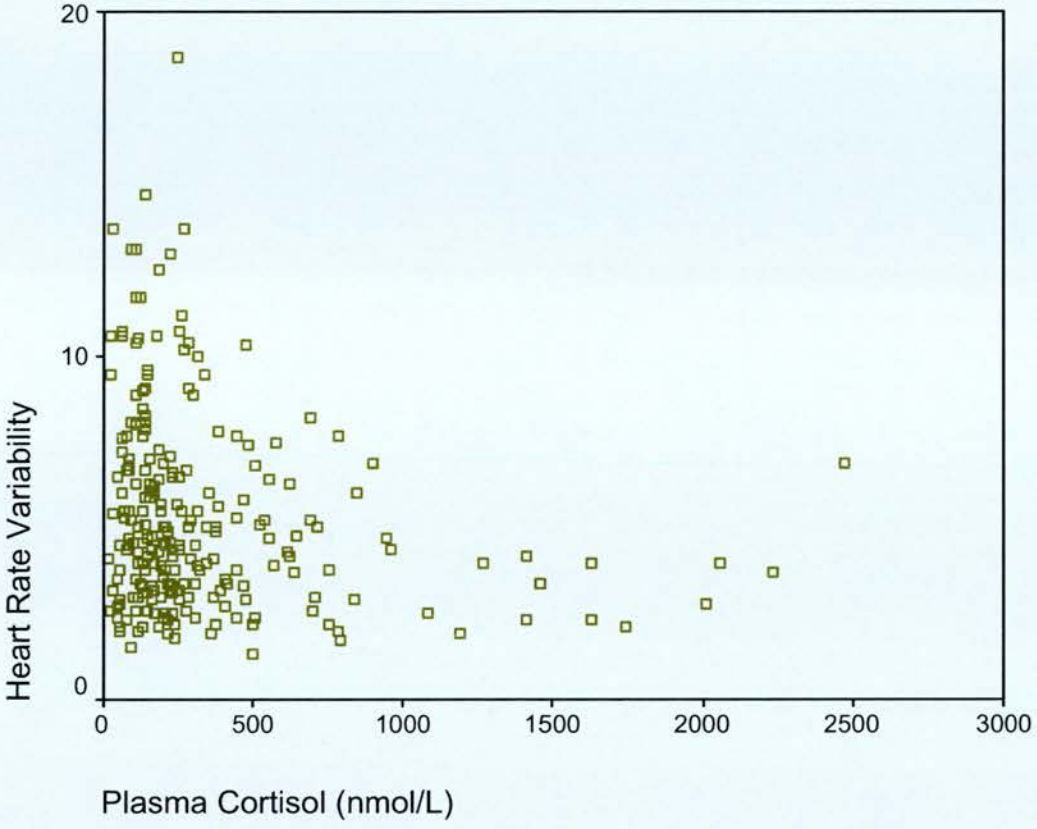
**Table 7.9:** Correlations between hormones and heart rate data

| | **Mean heart rate r** | **P value** | **Heart rate variability r** | **P value** |
|---|---|---|---|---|
| SP | 0.12 | 0.06 | -0.04 | 0.5 |
| NKA | -0.03 | 0.7 | -0.06 | 0.4 |
| Cortisol | 0.23 | **< 0.001** | -0.19 | **0.004** |

**Graph 7.5:** Scatterplot of mean heart rate and cortisol concentrations



**Graph 7.6:** Scatterplot of heart rate variability and cortisol concentrations




Surprisingly, assisted ventilation had little effect on mean heart rate. Mean heart rate was generally higher in ventilated infants, but this was only statistically significant on postnatal day 1 (Table 7.10). However, heart rate variability was a more useful marker of stress, as it was reduced as expected in ventilated infants, being statistically significant on postnatal days 1, 2 and 7 (Table 7.11).

Paradoxical results were obtained when AUC comparisons were made between ventilated infants who were randomised to morphine or placebo in the NEOPAIN subgroup (Table 7.12). It was anticipated that morphine would result in a reduction

in mean heart rate and an increase in heart rate variability, but a trend (not statistically significant) was observed in the opposite direction.

**Table 7.10:** The effect of assisted ventilation on mean heart rate, stratified by postnatal day

| Postnatal day | Ventilated | | Not ventilated | | P value |
|---|---|---|---|---|---|
| | N | Median mean HR | N | Median mean HR | |
| 1 | 35 | 148.2 | 21 | 143.3 | **0.02** |
| 2 | 33 | 155.9 | 18 | 148.1 | 0.06 |
| 3 | 32 | 156 | 18 | 150.4 | 0.1 |
| 7 | 18 | 160.1 | 19 | 157.7 | 0.9 |
| 14 | 11 | 160.1 | 12 | 160.4 | 0.8 |

**Table 7.11:** The effect of assisted ventilation on heart rate variability, stratified by postnatal day

| **Postnatal** | **Ventilated** | | **Not ventilated** | | **P value** |
|---|---|---|---|---|---|
| **day** | **N** | **Median HRV** | **N** | **Median HRV** | |
| 1 | 35 | 2.9 | 21 | 5.3 | **0.003** |
| 2 | 33 | 4.5 | 18 | 7.0 | **0.007** |
| 3 | 32 | 4.3 | 18 | 5.2 | 0.1 |
| 7 | 18 | 4.8 | 19 | 6.7 | **0.04** |
| 14 | 11 | 5.0 | 12 | 6.3 | 0.8 |

**Table 7.12:** Median AUC for heart rate data in ventilated infants on postnatal days 1 to 3, morphine versus placebo groups

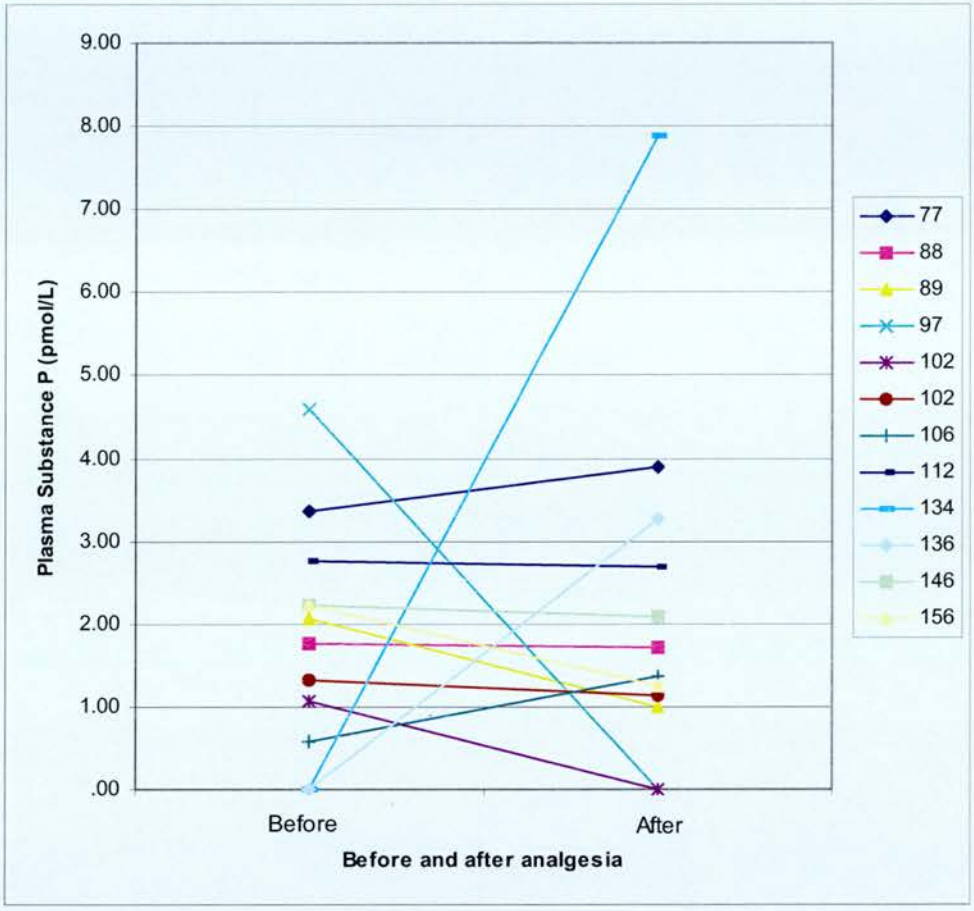
| | **Median AUC** | | **P value** |
|---|---|---|---|
| | Morphine | Placebo | |
| | *N=15* | *N=12* | |
| Mean heart rate | 313.9 | 307.1 | 0.4 |
| Heart rate variability | 7.2 | 8.4 | 0.7 |

## 7.7 LONGITUDINAL ANALYSIS OF CHANGES IN TACHYKININ AND CORTISOL CONCENTRATIONS WITH ANALGESIA

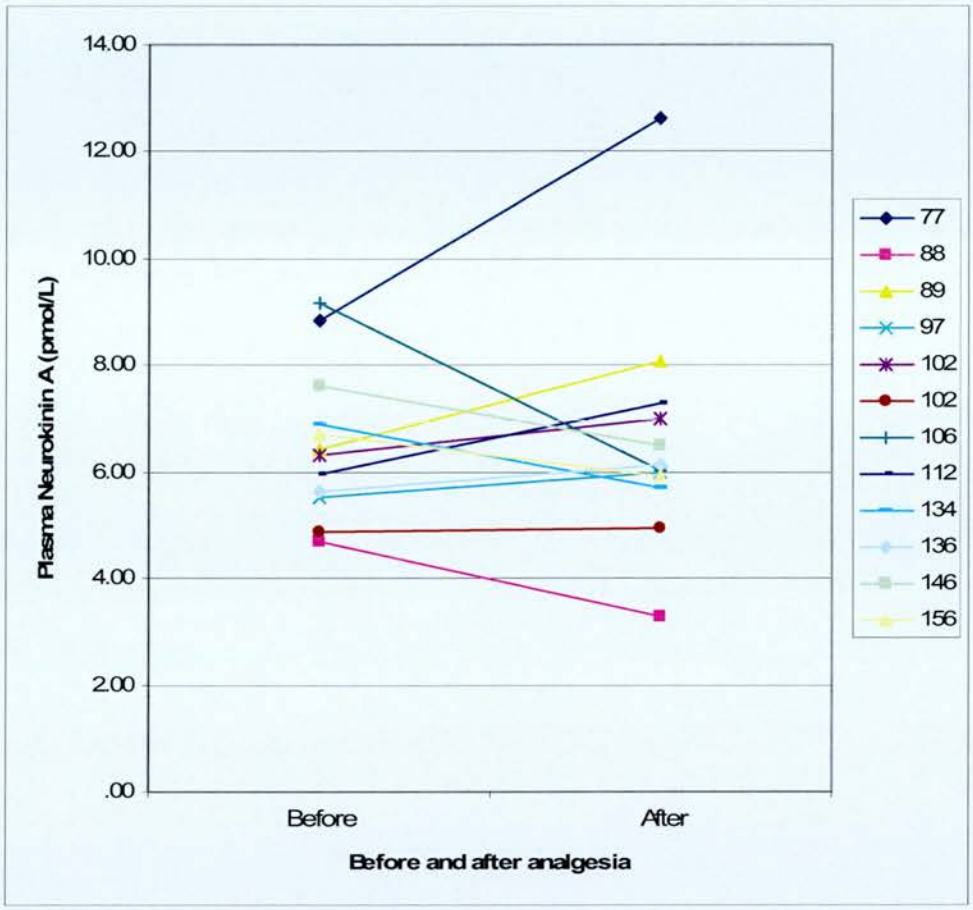
All data were examined for instances where successive tachykinin concentration results were available over time period where there was a change in the pain status of the infant. There were only in 2 instances in which tachykinin concentrations had been taken before and after an infant developed a painful condition (Section 7.2). However, there were 12 occasions in which plasma concentrations were measured before and after starting analgesia, whether it was for ventilation or for pain.

These changes are demonstrated in Graphs 7.7, 7.8 and 7.9. The mean percentage changes for neurokinin A and cortisol were +1.1% and –0.9% respectively, indicating that the changes occurred in either direction with analgesia. With substance P, the percentage change could not be calculated for 4 of the infants as they had substance P concentrations of 0.0 pmol/L. For the remaining 8 infants, the percentage change was +3.4%.

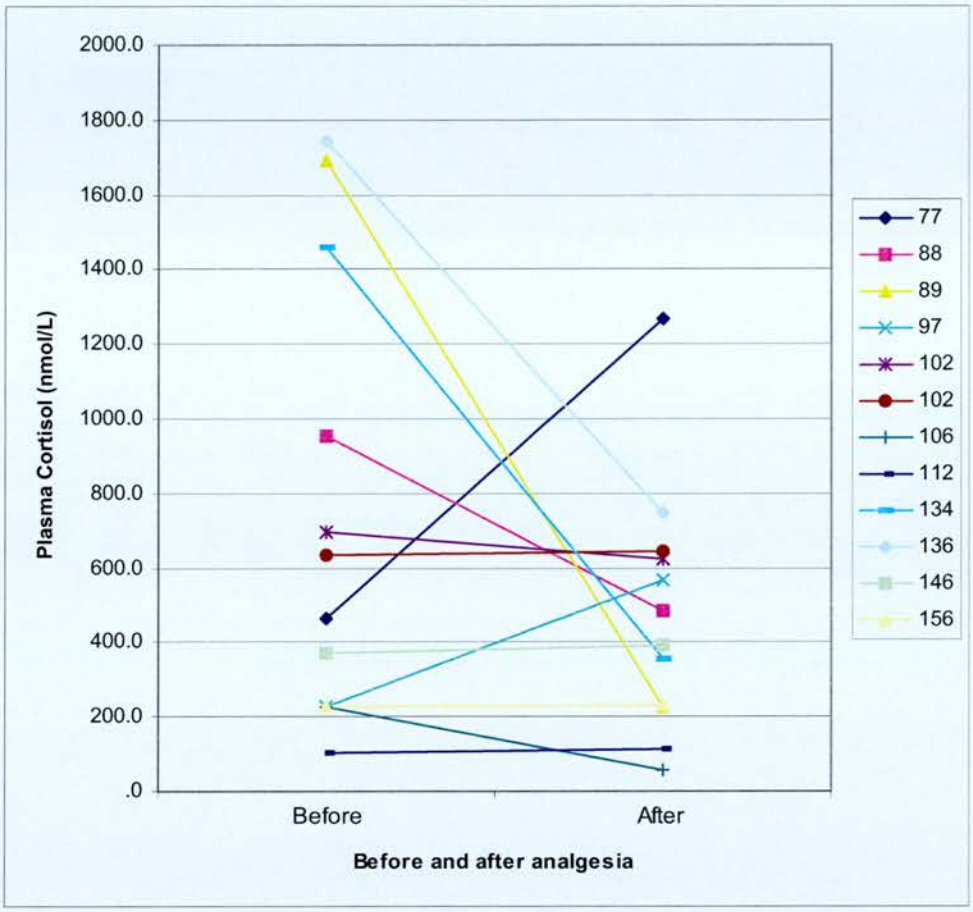
**Graph 7.7:** Change in plasma substance P concentrations with the administration of analgesia in 12 infants



**Graph 7.8:** Change in plasma neurokinin A concentrations with the administration of analgesia in 12 infants



**Graph 7.9:** Change in plasma cortisol concentrations with the administration of analgesia in 12 infants




## 7.8 Discussion

To determine whether SP and NKA concentrations varied with pain, a case-control study was set up and 19 infants thought to be in persistent pain were enrolled. There were no differences in plasma SP, NKA or cortisol concentrations between the pain group and their controls (Section 7.2). One major limitation of this observational study was that 15 of 19 infants believed to be suffering pain had already received analgesia prior to study enrolment, and it was possible that they were no longer in

pain. However, it is arguable on ethical grounds that the treatment of pain should not be withheld for any study. Analysis of the 4 who had not received prior analgesia yielded no significant differences in SP or NKA concentrations compared with either those who had received analgesia, or the controls.

Examination of plasma peptide concentrations over time for each infant within the 'pain' group also showed no consistent change with pain or analgesia administration (Section 7.2). However, in the whole cohort, there were 12 instances where infants had plasma samples taken before and after starting analgesia, whether for ventilation or for pain (Section 7.7). The percentage change was calculated for each of these occasions, but the mean percentage change was close to nil for each of the tachykinins, indicating that one could expect a change in plasma concentrations in either direction following the administration of analgesia. This longitudinal examination is limited by the fact that only 12 infants could be studied in this way.

Plasma cortisol was significantly higher in ventilated infants compared with non-ventilated infants, across different postnatal age groups (Table 7.3). Stratification across postnatal age was performed as plasma SP, NKA and cortisol concentrations were found to vary with postnatal age (Section 6.6). It is accepted that there are fundamental differences in the physiology of infants requiring ventilation versus those not requiring it. Infants who are more ill, e.g. with sepsis, are also more likely to need ventilatory support, and may have higher plasma cortisol concentrations as a non-painful stress response to their sepsis rather than as a pain response. Randomisation of newborn infants to ventilation or not is therefore not possible. For

example, an infant is more likely to require ventilation if it is more premature, and cortisol is higher in more premature infants (Section 6.4.1). Subanalysis of infants within gestational groups is difficult however, e.g. of 27 infants born ≤ 28 weeks' gestation, all but one were ventilated on days 1 and 2 of life in the current study. On day 3, 30 of 35 infants ≤ 28 weeks' gestation continued to require ventilatory support.

To eliminate the confounding factor of gestational age, the results were also analysed only for those infants ≤ 32 weeks' gestation (Table 7.3a). Despite there being smaller numbers, the differences between plasma cortisol concentrations of ventilated and non-ventilated infants remained highly significant.

Plasma SP concentrations yielded unexpected results, being not significantly different in ventilated infants (Tables 7.1 and 7.1a). As the original research hypothesis was that a rise in SP concentration was a manifestation of persistent pain in neonates, this finding leads to two possible deductions. Firstly, that production of SP in neonates is not upregulated in persistent pain, or secondly, that ventilation does not cause persistent pain to newborn infants. In fact, the question of whether plasma SP actually increases in painful conditions in adults (Section 1.5.1.4) has been raised again, now that sample extraction has been shown to be important for accurate determination of plasma concentrations (Chapter 2). Onuoha *et al* published three papers reporting raised plasma concentrations in adults with soft tissue injury[198], fractured necks of femur[214] and early burns[215], but did not use any form of plasma extraction. Marshall *et al* also reported increased plasma SP concentrations in adults with arthritis, but did not use plasma extraction either[138]. Finally, Molina also

published raised plasma SP concentrations in cases of spinal arachnoiditis, but did not provide any information on plasma extraction, sample handling, or assay type[199].

Conversely, those investigators who performed plasma extraction found *no* increase in SP in either acute pain (labour and post-operative[136]), or persistent pain (chronic headache[196], fibromyalgia[216], arthritis[192], chronic low back pain[137]). Hence it is feasible that plasma SP does not actually rise in response to persistent pain in either adults or neonates, although this does not negate the possibility that local upregulation in the central nervous system may occur.

Plasma NKA concentrations showed unexpected variation with ventilation, being significantly lower in ventilated infants on days 1 and 2, and higher on days 7 and 14 (Table 7.2). Even in the smaller subgroup of infants ≤ 32 weeks' gestation, this pattern was still significant on days 2 and 7, with a trend towards being significant on day 14 (Table 7.2a). Again, if one assumes the hypothesis that a rise in NKA concentration is a manifestation of persistent pain in neonates, the results on days 7 and 14 would be in keeping with the theory of assisted ventilation being a source of persistent pain. The next step to determining if this were true, would be to see if the administration of analgesia modifies the NKA response (Section 7.3). This was performed in the context of a randomised double-blinded placebo-controlled trial (NEOPAIN, Section 5.3.1.1). Administration of analgesia in the form of a continuous intravenous infusion of morphine resulted in a significantly lower plasma NKA concentrations, indicating that analgesia did indeed modify the NKA response. On the other hand, analgesia appeared to have no effect on plasma SP or cortisol

concentrations. This is probably because plasma SP was already shown not to rise in response to assisted ventilation in neonates, and whilst plasma cortisol may be a good marker for non-painful stress, it may be a less specific marker for persistent pain.

The unexpected results of plasma NKA concentrations being significantly lower in ventilated infants on days 1 and 2 may be related to the effect of perinatal factors (Section 6.6). Ventilated infants were more likely to have undergone labour, been born by spontaneous vertex delivery, and their mothers not have epidural analgesia, all factors which were associated with lower plasma NKA concentrations on day 1, although the only statistically significant difference was with regard to labour (Table 7.13).

**Table 7.13:** Differences in perinatal factors between ventilated and non-ventilated infants

| **Antenatal factors** | **Ventilated N (%)** | **Not ventilated N (%)** | **P value** |
|---|---|---|---|
| Labour | 32/39 (82%) | 16/31 (52%) | 0.01 |
| SVD | 21/39 (54%) | 10/31 (32%) | 0.07 |
| No epidural analgesia | 24/39 (62%) | 13/31 (42%) | 0.07 |

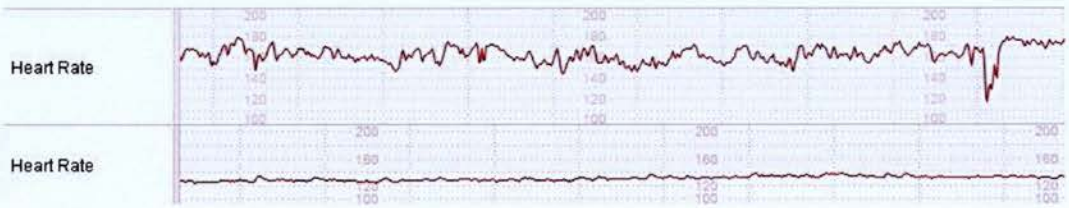
The PIPP multimodal infant pain assessment tool was used to obtain a measurement of the degree of pain the infants may have been in using behavioural and physiological indicators (Section 1.4.1.4). The difficulty with using PIPP scoring is that it has been validated for acute procedural pain such as venepuncture, but not for persistent pain. Resting scores have only recently been investigated and validated for post-operative pain assessment[49]. Scores in response to oral suction have not been validated before. However, as most of the ventilated infants had blood samples taken via an indwelling arterial line, pain stimulus scores would not be available for this important group of infants, hence suction scores were measured as oral suction was a standard intervention undergone by all infants for the collection of saliva.

There was no difference between ventilated and non-ventilated infants with regard to their suction and pain stimulus PIPP scores. Resting PIPP scores showed a statistically significant difference between ventilated and non-ventilated infants on postnatal days 1 and 2, but the actual difference of 1 point in the score was clinically insignificant (Table 7.6). Suction PIPP scores were generally higher in non-ventilated infants, though not statistically significant (Table 7.7). This could be due to the fact that PIPP scores include assessment of a facial grimace, which may be less visible in an infant with an endotracheal tube *in-situ*. However, a lack of facial grimace could also be an indication of persistent pain. Unfortunately the number of ventilated infants who had a PIPP score performed with a painful stimulus was very small, making interpretation of those results very difficult (Table 7.8).

Plasma SP showed no correlation with PIPP scores, though this is unsurprising as SP was shown not to rise in response to assisted ventilation (Table 7.5). Plasma NKA showed a strongly significant positive correlation only with pain stimulus PIPP scores, despite there being fewer infants with pain stimulus PIPP scores than resting or suction scores. This may indicate that the infants who reacted most strongly to procedural pain already had some upregulation of their background pain mechanisms. Plasma cortisol showed a significant negative correlation with suction and pain stimulus PIPP scores, which may be because sicker infants who are under more physiological stress are more likely to exhibit frozen facial appearance and behaviour[42].

Heart rate and its variability were also used as other proxy measures of pain. Figure 7.1 shows Badger System® screenshots comparing normal and reduced heart rate variability graphical trend displays.

**Figure 7.1:** Badger System® screenshots comparing normal (top) and reduced (bottom) heart rate variability graphical trend displays



Although mean heart rate was generally higher in ventilated infants, this was only statistically significant on postnatal day 1 (Table 7.10). However, a reduced heart rate variability appeared to be more reflective of the degree of stress experienced by ventilated infants (Table 7.11). Reduced heart rate variability has already been shown to be indicative of neonatal sepsis and systemic inflammation[217], and to be associated with neonatal mortality[218]. It is also seen antenatally on cardiotocograms as an indication of fetal stress *in utero*[219;220]. In adults, reduced heart rate variability after myocardial infarction is prognostic of reduced long-term survival[221]. It is therefore unsurprising that plasma cortisol, an established marker of stress, shows a strongly significant positive correlation with mean heart rate, and negative correlation with heart rate variability (Table 7.9). Disappointingly, neither plasma SP nor NKA showed any correlation with heart rate or its variability.

It is more difficult trying to delineate whether the reduced heart rate variability in ventilated infants is due to the stress of pain, or of being sicker and consequently needing ventilatory support. If the heart rate variability was reduced because of pain, then administration of analgesia to ventilated infants should reverse the phenomenon of decreased variability. However, this is not the case (Table 7.12). Heart rate variability was not modulated by analgesia, as was found to be the case for plasma cortisol. Both plasma cortisol and heart rate variability therefore appear to be good markers for non-painful stress, but not for persistent pain.

# 8. CLINICAL STUDY: SALIVA SUBSTANCE P AND NEUROKININ A IN NEONATES

## 8.1 Introduction

The aim of investigating SP and NKA in saliva samples was to find a less invasive alternative to blood sampling. This would require SP and NKA concentrations to be mirrored between plasma and saliva samples.

296 saliva results were obtained, compared with 485 plasma results. The remaining 189 samples were either insufficient (ie. empty) or missing. Saliva samples were completely missing from (i) all five infants enrolled from the Royal Hospital for Sick Children, Edinburgh, (ii) subjects no. 70 to 97, and (iii) subjects no. 127 to 136. Consequently, only 131 of the cohort of 174 infants had saliva results, and of these 131, 107 infants had incomplete results due to insufficient or missing samples on various days.

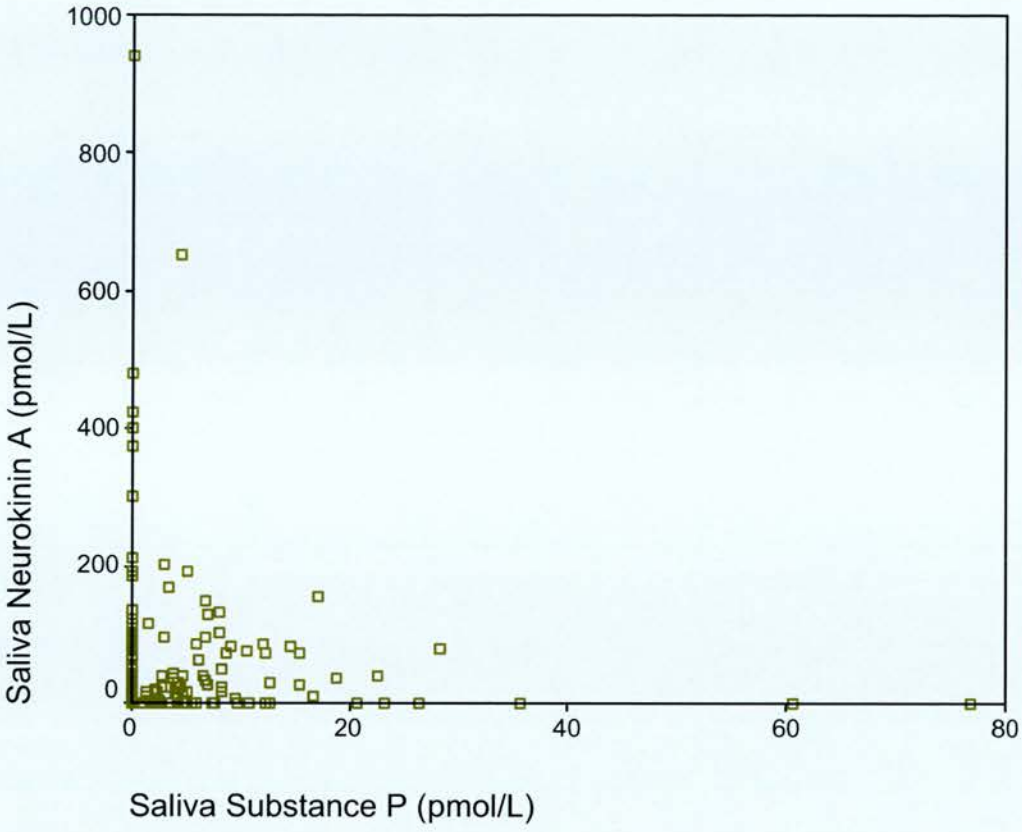
Of the 296 saliva samples analysed, 161 (54%) were ≤ 50 μl, 64 (22%) were ≤ 20 μl, and 42 (14%) were ≤ 10 μl. This was due to low saliva yields in the infants. By comparison, only 8 of 485 plasma samples were ≤ 50 μl (0.02%). Furthermore, a poor NKA standard curve was encountered during radioimmunoassay analysis of samples from subjects no. 144 to 170. A total of 93 NKA results were therefore rendered unreliable. However, this still left 203 saliva NKA results for interpretation.

In view of the multiple difficulties encountered with saliva sampling, and the consequent poor and patchy yield in results, only limited statistical data analysis was performed.

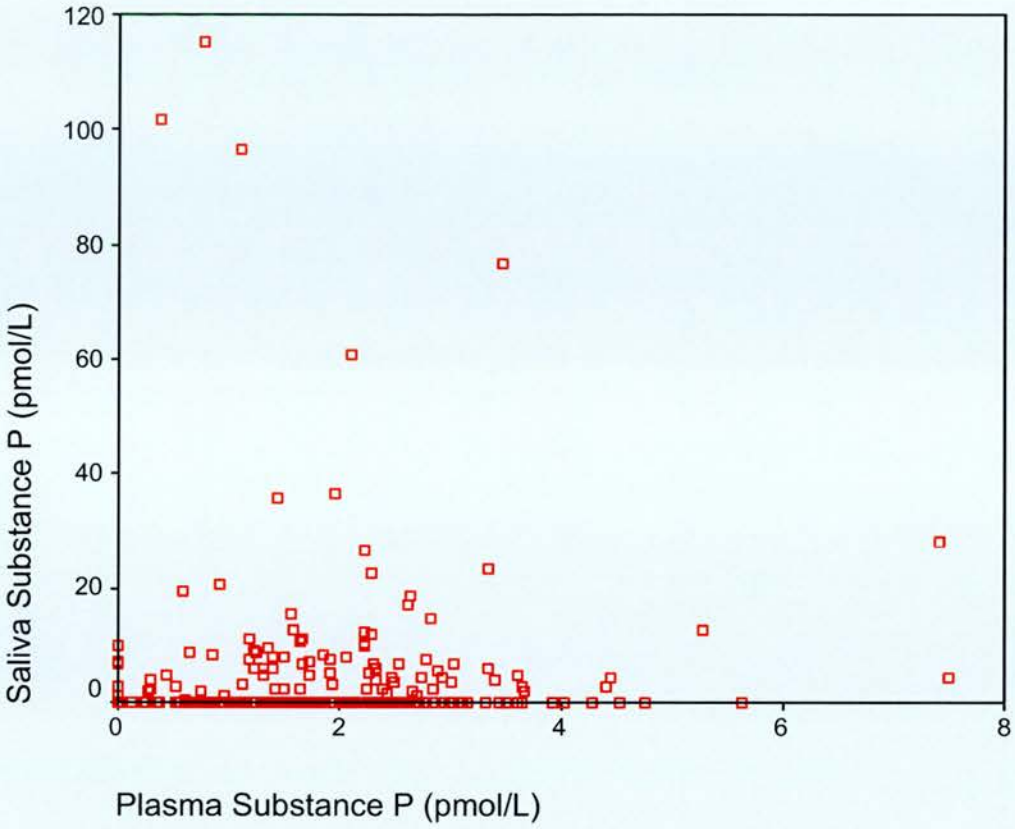
## 8.2 CORRELATION BETWEEN SALIVA HORMONES, AND WITH PLASMA EQUIVALENTS

There was statistical correlation between saliva SP and saliva NKA ($r = 0.20$, $P = 0.005$). However, inspection of the actual scatterplot for these results showed that there was no clinically useful relationship between the two salivary hormones (Graph 8.1). A considerable number of samples yielded high concentrations of NKA with no detectable concentrations of SP, and vice versa. Saliva SP correlated weakly with plasma SP ($r = 0.14$, $P = 0.016$) (Graph 8.2) but saliva NKA did not correlate at all with plasma NKA ($r = 0.02$, $P = 0.8$) (Graph 8.3).

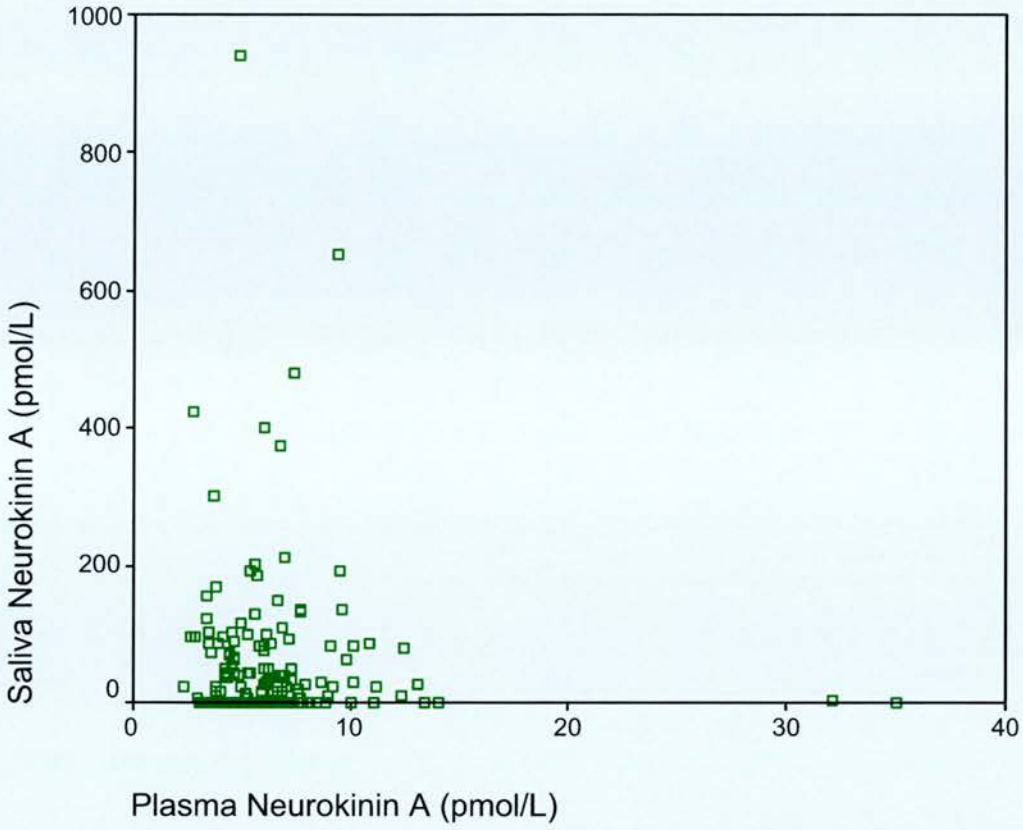
**Graph 8.1:** Scatterplot of paired saliva NKA and saliva SP concentrations



**Graph 8.2:** Scatterplot of paired saliva SP and plasma SP concentrations



**Graph 8.3:** Scatterplot of paired saliva NKA and plasma NKA concentrations




## 8.3 BASELINE CONCENTRATIONS OF SALIVA SP IN NEONATES

As with plasma SP, there was an overall skewed distribution of saliva SP concentrations. For infants not in pain, these ranged from 0.0 to 115.16 pmol/L, an appreciably larger range than for plasma SP concentrations. However, 198 samples yielded a result of 0.0 pmol/L (67%), and the median saliva SP concentration was therefore 0.0 pmol/L. The large number of samples yielding this result could be due to sample volumes being small. Furthermore, only 18 results were ≥ 15 pmol/L, and of these, 16 were from sample volumes that were 50 μl or less, illustrating that small

sample volumes can also lead to large errors due to the multiplication factors involved after sample extraction.

Saliva SP also showed a weak statistical correlation with gestation ($r = 0.25$, $P = 0.02$) and birth weight ($r = 0.22$, $P = 0.04$), but again, the scatterplots revealed no important association.

## 8.4 Baseline concentrations of saliva NKA in neonates

Saliva NKA also demonstrated an overall skewed distribution. For infants not in pain, concentrations ranged from 0.0 to 942.04 pmol/L, again a much larger range than for plasma NKA. Unlike plasma NKA, which was always detectable, 102 saliva samples yielded an NKA result of 0.0 pmol/L (50%), making the median saliva NKA concentration also 0.0 pmol/L. Again, only 9 results were ≥ 200 pmol/L, and 7 of these were 10 μl samples.

Saliva NKA showed no correlation with gestation ($r = 0.22$, $P = 0.07$) or birth weight ($r = 0.19$, $P = 0.12$).

## 8.5 Discussion

Saliva sampling proved challenging in newborn infants. It was often noted that saliva production was scanty, and this was reflected by the poor yield in results. It is also difficult to distinguish gingival crevicular fluid and mucus from saliva in neonates. In fact, it is probably more accurate to call it oral fluid sampling, rather than saliva sampling. Furthermore, copious amounts of oral fluid were obtained from babies

who were paralysed with pancuronium or vecuronium, which could cause potential bias in results.

A number of methodological problems must be acknowledged here. Despite intensive efforts to trace them, all saliva samples from 43 infants went missing from the laboratory. This included samples from 13 of the 19 infants thought to have been in pain. It was also disappointing that a poor NKA standard curve rendered 93 saliva NKA results unreliable.

The high number of missing or insufficient results meant that most infants had incomplete data, hence variation of saliva SP and NKA with postnatal age could not be investigated. Correlation between the saliva neuropeptides, and with their plasma equivalents, could be performed, but this only showed a weak relationship between saliva and plasma SP (Section 8.1).

Although saliva SP concentrations appeared to be detectable at a higher range than plasma SP (up to 115.16 pmol/L versus 11.2 pmol/L), the median saliva SP concentration was in fact 0.0 pmol/L, compared with 1.7 pmol/L in plasma (Section 8.2). This is because 198 (67%) of 296 saliva samples yielded a result of 0.0 pmol/L for SP. The high concentrations were from sample volumes that were very small, and probably resulted from the large multiplication factors required after sample extraction. Overall, therefore, saliva SP existed in lower concentrations than plasma SP in neonates, which is contrary to findings in adults[137].

The same was true for saliva NKA concentrations. Although the range of NKA concentrations extended to 942.04 pmol/L in saliva, versus a maximum of 74.6 pmol/L in plasma, the median saliva NKA concentration was also 0.0 pmol/L. This is compared with a median plasma NKA concentration of 6.0 pmol/L.

It is possible that a large number of saliva SP and NKA results yielded a value of 0.0 pmol/L because there was not sufficient saliva in the oral fluid collected. It could also be that SP and NKA exist in saliva in concentrations below the limit of detection of the radioimmunoassays. Another conclusion might be that the salivary glands of the neonate do not produce SP and NKA in significant quantities, even under circumstances which might be considered painful or stressful. Further work requires to be done to test this hypothesis.

# 9. SUMMARY AND CONCLUSIONS

In neonatal intensive care, it is a daily challenge to ascertain whether infants requiring ventilatory support are experiencing pain, and if so, to determine how much pain they are in and whether it is alleviated by analgesia. Infants are preverbal and hence cannot describe their discomfort. The sickest and most premature infants are also the ones least able to communicate their pain and distress. Yet, there is evidence that infants can feel pain, and that they are able to mount an endocrine and metabolic stress response to it. This response can be detrimental to the infant, by promoting a catabolic state, water retention, hyperglycaemia, and impairing bowel and immune function. Wound healing is impaired and susceptibility to infection is increased, together with mortality.

The ability to measure pain in infants undergoing neonatal intensive care, as well as to monitor responses to analgesia, would facilitate more efficient pain management. Behavioural measures are the mainstay of infant pain assessment, but these have principally been devised for assessing acute rather than persistent pain. Such techniques also cannot be used when an infant is paralysed, which is sometimes necessary in the sickest infants. Physiological measures, such as heart rate, are affected by many other factors, e.g. infection, drugs, and simple handling. Many existing neurochemical measures are markers for non-painful stress rather than pain. This series of studies has served to confirm this for both plasma cortisol and heart

rate variability (Sections 7.4 and 7.6). There is therefore the ongoing need to find a suitable marker for pain that could eventually be developed for clinical use. There is currently no gold standard measure for persistent pain in newborn infants, but that should not preclude the study into possible future markers of pain.

Worldwide, these are the first studies of SP and NKA in newborn infants. Plasma SP and NKA concentrations in newborn infants are described across various gestational ages and changes with postnatal age are investigated. It is also the first to examine whether concentrations of SP or NKA are affected by persistent pain, assisted ventilation, or the administration of analgesia.

To conduct this study with rigour, methods of sample handling and laboratory analysis to maintain neuropeptide stability and to ensure result validity were scrutinised (Chapters 3 and 4). A significant number of previous publications on *in vivo* studies of SP have reported that concentrations of SP vary with different clinical conditions, but where these have failed to employ the use of sample extraction, their findings have now been thrown into doubt. Even existing radioimmunoassay kits for analysing adult plasma samples have quoted extraction procedures that are suboptimal, resulting in little or no recovery of the relevant neuropeptide.

Peptide extraction was found to be essential for the accurate analysis of plasma SP and NKA (Chapter 2). Both liquid phase and solid phase extraction procedures have been evaluated in the current studies. Different solid phase extraction columns and mobile phases were also tested. It was important that a single extraction procedure

yielded high recoveries for both neuropeptides, as both SP and NKA concentrations needed to be measured in each sample.

Apart from sample extraction, the radioimmunoassays for both neuropeptides also needed to be modified for analysis of neonatal microsamples (Chapter 3). The most specific antibody was chosen, then ideal antibody concentrations determined. The stability of standards was checked. Disequilibration of the assays and different second antibodies made little difference to the assay.

Plasma SP concentrations found in this study (median 1.7 pmol/L) were similar to those found in other adult studies that employed sample extraction prior to assay (Section 6.2). For example, Joyce *et al*[192] used enzyme inhibitors, a validated extraction procedure, HPLC coupled with RIA, and found that SP concentrations in plasma and synovial fluids in patients with arthritis were approximately 1000-fold less than those previously reported by others using less stringent analytical techniques, e.g. Marshall *et al*[138]. As in this study, Joyce found that unextracted plasma and synovial fluid gave higher measurements than extracted samples. The only other study on plasma SP in infants was in babies over the age of three weeks, and no sample extraction was performed[190]. It is therefore likely that these results are a more accurate reflection of true plasma SP concentrations in newborn infants.

Plasma SP concentrations were found to be very low, and often undetectable, despite the use of a rigorous extraction technique and a modified assay for increased sensitivity (Section 6.2). Plasma NKA concentrations existed in higher amounts than

plasma SP (median 6.0 pmol/L) (Section 6.3). This may not be surprising as there is evidence from neurobiological studies that SP exerts its effects locally at the site of stimulation of primary afferents, whilst NKA activates more distant sites[117]. SP may therefore tend to be a more useful marker of upregulation in nociceptor activity if measured locally, e.g. in synovial fluid in arthritis, or in the CSF in headaches and other painful spinal diseases. It is maybe therefore unsurprising that different researchers found conflicting results when studying the changes in plasma SP with chronic pain of various origins (Section 1.5.1.4). Also, it would be even less likely for changes in salivary SP to be perceived if plasma SP already shows such a low level of detection and little measurable change. NKA is more likely to be detected systemically if it exerts its effects at more distant sites.

The current studies show that gestation and birth weight have no effect on plasma SP or NKA (Chapter 6). Plasma SP and NKA show changes with postnatal age, more marked in preterm infants. The slight increase in the first three postnatal days and subsequent fall back to baseline seems in line with other pain/stress regulators, e.g. catecholamines. In early postnatal days, multiple factors may influence SP and NKA concentrations, including perinatal circumstances. This is seen in unexpectedly higher plasma NKA concentrations in the absence of labour, in infants born by caesarean section, and if maternal epidural analgesia was administered. The mechanisms behind this apparent upregulation of NKA are unclear, and further research on NKA concentrations in umbilical cord blood samples may shed light on this, as they would help differentiate changes in concentrations due to postnatal

events versus perinatal factors. Perinatal factors did not appear to affect plasma SP concentrations.

In these studies, persistent pain did not appear to have a significant effect on concentrations of either neuropeptide (Section 7.2). It is possible that some or all of the infants were not in persistent pain as was assumed. However, it may also be due to adequate treatment of pain with analgesia administration. Only 4 of the 19 infants in pain did not receive analgesia. Then again, the appropriate treatment of pain should not be withheld for any study. It was more feasible to study SP and NKA concentrations in a population of infants who were not routinely receiving analgesia, but exposed to another source of possible pain or distress, such as assisted ventilation. This was investigated opportunistically with infants involved in the NEOPAIN study (Section 7.4).

The results of the NEOPAIN study have now been published[193]. A total of 898 ventilated infants were enrolled from 16 centres with equal division into morphine and placebo arms of the trial. Pre-emptive morphine infusions did not reduce the frequency of severe IVH, PVL, or death in ventilated preterm neonates, but intermittent boluses of open-label morphine were associated with an increased rate of the composite outcome. The morphine doses used in the study decreased clinical signs of pain but also caused significant adverse effects in ventilated preterm neonates.

Prolonged opiate administration may also produce unwanted pro-nociceptive adaptations which result in hyperalgesia[222]. SP and NK1R expression is also increased in the spinal dorsal horn during sustained opiate therapy. Hence the use of prolonged opiate infusions in the NEOPAIN study may have resulted in an upregulation of SP concentrations and therefore a false negative in finding a decreased concentration with analgesic therapy.

With regard to either pain or assisted ventilation, plasma SP concentrations did not appear to be a useful marker of persistent pain or distress. Conversely, plasma NKA concentrations did not vary with pain, but showed significant changes with ventilation, which were further modulated by the use of analgesia. This was seen on the cross-sectional analysis of results (Section 7.4). However, in a small subgroup of infants, there was the opportunity to study data longitudinally (Section 7.7). In this subgroup, the mean percentage change in plasma neuropeptide concentrations was close to nil, indicating that changes could occur in either direction with the administration of analgesia. However, this was studied in only 12 infants, hence the cross-sectional data comparing 31 infants may be more representative.

In the cross-sectional analyses, cortisol responses in the same group of infants demonstrated significant changes with ventilation, but not with analgesia. This suggested that although cortisol is a useful indicator of overall non-painful stress, NKA might be more specific for pain.

The current studies found a weak correlation between plasma SP and NKA (Section 6.5). Given that SP and NKA are generally co-synthesised and co-secreted when the PPT-A gene is switched on, a correlation is not unexpected. That it is a weak one, however, probably confirms again that the two peptides are different in their biological functions, activity and areas of action (Sections 1.5.1.3 and 1.5.2.2).

Plasma SP did not correlate with plasma cortisol or other physiological measures of pain used in this study (Sections 6.5, 7.5 and 7.6). Plasma NKA did not correlate with plasma cortisol, but showed some correlation with pain stimulus PIPP scores only. This could be due to a true lack of change in plasma SP and NKA with pain, but could also be due to the physiological and behavioural measures themselves being inadequate measures of persistent pain. As mentioned in Section 1.4.1.4, PIPP scores have been validated mainly for use with acute pain, while the infants in this study were exposed to more persistent pain. Yet the PIPP score was chosen as the best possible behavioural measure of persistent pain because it is the only measure which has been validated for use in more persistent pain, i.e. post-operative pain. The results from these studies give some indication that the PIPP may still not be an adequate score for the assessment of chronic pain. There is therefore still a need for better clinical assessment of chronic pain in infants.

Heart rate variability was found to be a better marker of stress in ventilated infants than PIPP scoring (Section 7.6). PIPP scoring in a premature infant, although predominantly a behavioural marker with a more 'emotional' component than a chemical marker, is nevertheless still likely to be more a measurement of nociceptive

circuit reflex behaviour rather than a true emotional pain response. Neither neuropeptide correlated with heart rate variability, whereas plasma cortisol did. As with plasma cortisol, heart rate variability was not modulated by the use of analgesia, suggesting that both were markers for non-painful stress but not necessarily for pain. There is therefore still no gold standard measure for the assessment of chronic or persistent pain in newborn infants, especially premature ones. Chronic pain which is of sufficient importance would be that which leads to physiological or psychological maladaptation, or neurobiological 'rewiring' and subsequent hyperalgesia, allodynia or dysaesthesia. Perhaps the only way of determining which infants suffer this would be to assess their pain responses on long-term follow-up, perhaps even into adulthood if necessary.

Understanding the neurobiology of pain transmission in premature infants also makes us realise that it is not only painful stimuli that need treating, but also other tactile stimuli (Section 1.1.4). Receptive fields in neonates are larger and more disorganised, and in premature infants hyperstimulation with a lack of local regulation may have noxious consequences. Neurotrophin concentrations themselves are partly responsible for nociceptor innervation density in the skin and can be upregulated by neonatal skin wounding[5]. Unfortunately, premature infants are subject to multiple skin wounds during the course of their intensive care, and this can result in local upregulation of neurotrophins in the skin, possibly also detected in plasma samples. With adequate treatment of these repeated skin wounds, the nociceptor innervation density may possibly be normalised, although the evidence

for this from animal studies is still awaited. We now know it is important to treat neonatal pain to avoid subsequent abnormal pain reception or behaviour[14].

So, where to from here? Will there ever be a reliable behavioural, let alone neurochemical, measure of pain in newborn infants to be found? Even if a neurochemical marker is discovered, it is more likely to be measure of nociceptor pathway upregulation rather than pain. However, if it proves to be a reliable measure of persistent pain, development of a bedside assay would allow it to be used in clinical care.

In this pilot study, the findings with regard to NKA suggest further investigation is warranted. SP appears to be detected in plasma at concentrations too low to ascertain any variation, and perhaps should be measured at a local rather than systemic level. The unexpected variations of NKA with labour, caesarean section, and maternal epidural analgesia may best be examined by comparing cord blood samples of neuropeptide concentrations with day 1 samples. The day 1 samples in this study were taken anytime in the first 24 hours of an infant's life, hence could have been influenced by postnatal treatments already. How true these associations of NKA with perinatal factors are is therefore unclear, and could be elucidated with cord blood samples.

Plasma NKA could be perhaps be studied in an older group of verbal children over a time when pain is being experienced, and in the recovery phase. This could comprise children who are pre- and post-operative, and correlated with a verbal or pictorial

pain scale. However, if these children receive adequate peri-operative analgesia, the same problems could be encountered as in this study, where measurements were probably taken in the absence of any pain sensation due to adequate therapy. An alternative option would be to study children who have sustained a painful insult, which is likely to be persistent, such as a bone fracture, and measure samples when they are in pain and during the recovery period, again with a descriptive pain scale for correlation.

These potential studies would be in older verbal children, to look for a relationship between the neurochemical marker and the emotional sensation of pain. If found, the results could be extrapolated to children of a similar age who may be non-verbal. However, we would still have to be cautious in interpreting similar results in newborn infants, as the neurobiology and neurophysiology is likely to be very different.

Due to having an immature nervous system, newborn infants experience pain via functional signalling pathways that are not found in mature individuals. Pain signalling occurs via a complex interaction of neurotransmitters and neuromodulators at different levels in the nervous system. As such, it is unlikely that any one neurotransmitter can serve as a 'marker' for pain, and that perhaps a measure of variations in a selected set of neurotransmitters may be more informative. Furthermore, there is some evidence from animal studies that A fibres are also involved in rapid nociceptive transmission in the newborn, especially premature, and hence should also be investigated together with C fibre nociception[4]. However, as the

study of the developmental neurobiology of pain signal processing advances, there is hope that analgesic therapies which are more specific to newborn infants will be developed in the future.

## Appendix A: Materials

### Laboratory Work

### Hardware

| Abbreviation | Full name | Manufacturer | Serial no. | Equipment no. |
|---|---|---|---|---|
| Glass SPE tank | IST VacMaster | IST | | |
| Second SPE tank | Vac-Elut | Analytichem International | | |
| Gamma counter | Cobra II Auto-gamma | Packard Canberra | 403042 | 04787 |
| Cold fuge | CR422 | Jouan France | 29412269 | 04785 |
| Orange vortex mixer | Whirlimixer | Fisons | | 04786 |
| White vortex mixer | Autovortex Mixer SA2 | Stuart Scientific | 6720 | 04792 |
| Water bath | W38 | Grant | | 04796 |
| Magnetic stirrer | S/MAG/MINOR | Voss Instruments Ltd. | | 06203 |
| Microfuge | MBC Microfuge | Hawksley England | | 04721 |
| pH meter | pH Meter 240 | Corning | | 04724 |
| Pipettes | Scalpettes (various) | Jencons | | |
| Pipettes | Proline (various) | BioHit | | |
| Vacuum pump | Aquavac Junior | Aquavac Uniscience | 769 | 04793 |
| Gyrovap | Gyrovap | Howe & Co. UK | GV1623 | 04681 |
| Spectrophotometer | Utrospec II | LKB Biochrom | 03033 | 04624 |

## Consumables

Phosphate Buffer

| Abbreviation | Full name | Manufacturer | Product no. | Lot no. | Notes |
|---|---|---|---|---|---|
| Acid phosphate | Sodium dihydrogen orthophosphate (1-hydrate) | BDH AnalaR | 102454R | A191821 | FW=137.99 |
| Alkaline phosphate | di-Sodium hydrogen orthophosphate (anhydrous) | BDH AnalaR | 102494C | F1018981 | FW=141.96 |
| EDTA | Ethylenediamine-tetraacetic acid, disodium salt dihydrate | Sigma-Aldrich | 25,235-2 | 77518 | FW=372.24 |
| Na azide | Sodium azide 99% | Sigma-Aldrich | 19,993-1 | 40105 30610079 | FW=65.01 |
| Trasylol | Aprotinin | Bayer | | CBVWU1 CBNPR1 | |
| Albumin | Human albumin fraction V | Sigma-Aldrich | A-1653 | 40K7606 | |

Liquid Phase Extraction Procedure

| Abbreviation | Full name | Manufacturer | Product no. | Lot no. | Notes |
|---|---|---|---|---|---|
| Ace | Acetone | BDH AnalaR | 100035R | K27304306 | 58.08 |
| PetEt | Petroleum spirit 40-60°C | BDH AnalaR | 10178 | 3378600M | |
| Nitrogen | Nitrogen $N_2$ (oxygen-free) | BOC Gases | 1066 | | |

Solid Phase Extraction Procedure

| Abbreviation | Full name | Manufacturer | Product no. | Lot no. | Notes |
|---|---|---|---|---|---|
| C18 cartridges | Isolute SPE columns C18 50mg/1ml | IST | 220-0005-A | | |

Fehder Mobile Phase

| Abbreviation | Full name | Manufacturer | Product no. | Lot no. | Notes |
|---|---|---|---|---|---|
| MeOH | Methanol | BDH AnalaR | 10158BG | K26829270 | 32.04 |
| HAc | Acetic acid | BDH AnalaR | | | |
| ACN | Acetonitrile | BDH HiPerSolv | 152856K | 1904310 | 41.05 |
| TFA | Trifluoroacetic acid about 100% | BDH HiPerSolv | 153112E | 8436290P | 114.02 |
| Nitrogen | Nitrogen $N_2$ (oxygen-free) | BOC Gases | 1066 | | |

Bachem Mobile Phase

| Abbreviation | Full name | Manufacturer | Product no. | Lot no. | Notes |
|---|---|---|---|---|---|
| TFA | Trifluoroacetic acid about 100% | BDH HiPerSolv | 153112E | 8436290P | 114.02 |
| ACN | Acetonitrile | BDH HiPerSolv | 152856K | 1904310 | 41.05 |
| Nitrogen | Nitrogen $N_2$ (oxygen-free) | BOC Gases | 1066 | | |

Substance P Radioimmunoassay

| Abbreviation | Full name | Manufacturer | Product no. | Lot no. | Notes |
|---|---|---|---|---|---|
| SP Antibody | | IDS/Eurodiagnostica | A45 | SP-2-840530 | |
| SP Tracer | | | | | |
| SP Standard | | Peninsula | 7451 | | |
| SP Standard | | Sigma | S6883 | | |
| Sac-Cel | Solid phase second antibody-coated cellulose | IDS | AA-SAC1 | 39622 41200 | |

| | | | | | |
|---|---|---|---|---|---|
| | suspension (anti-rabbit) | | | | |
| DARS/NRS | Donkey anti-rabbit serum/normal rabbit serum | Scottish Antibody Production Unit | | | |

Neurokinin A Radioimmunoassay

| **Abbreviation** | **Full name** | **Manufacturer** | **Product no.** | **Lot no.** | **Notes** |
|---|---|---|---|---|---|
| NKA Antibody | | Peninsula | RIK7359 | | |
| NKA Tracer | | | | | |
| Sac-Cel | Solid phase second antibody-coated cellulose suspension (anti-rabbit) | IDS | AA-SAC1 | 39622 41200 | |
| DARS/NRS | Donkey anti-rabbit serum/normal rabbit serum | Scottish Antibody Production Unit | | | |

Miscellaneous

| | | | | | |
|---|---|---|---|---|---|
| Polypropylene tubes | | | | | |
| Polystyrene RIA tubes | | | | | |
| Pipette tips | | | | | |
| Pastettes | | | | | |

**Clinical Study**

| **Abbreviation** | **Full name** | **Manufacturer** | **Serial no.** | **Equipment no.** |
|---|---|---|---|---|
| NNU microfuge | Micro-haematocrit centrifuge | Hawksley England | 1500 | 483202 |
| Eppendorf tubes | | | | |

| | | | | |
|---|---|---|---|---|
| EDTA | Ethylenediamine-tetraacetic acid, disodium salt dihydrate | Sigma-Aldrich | 25,235-2 | 77518 |
| Trasylol | Aprotinin | Bayer | | CBVWU1<br>CBNPR1 |
| Syringes | 2 ml Omnifix LuerLock<br>1 ml LuerLok | Braun Germany<br>BD Singapore | 4617029V<br>301283 | |
| Needles | Microlance sterile needles | BD International | | |
| Sterets | | | | |
| Pastettes | | | | |
| Suction catheter | Graduated suction catheter FG10 | Meddis Oxfordshire | CT02.10.035 | |
| Suction trap | Tracheal suction set | Maersk Denmark | 24001182 | |
| Saliva tube | | | | |
| Transpore | Transpore surgical tape | 3M USA | 1527-1 | |
| Badger system | Badger Patient Data Management System | Clevermed | | |
| Siemens monitors | Infinity modular monitoring series | Siemens USA | SC7000 | |
| ECG leads | Blue Sensor ECG electrodes | Medicotest Denmark | BR-50-K<br>BRS-50-K | |
| Arterial BP kits | Pressure monitoring set | Edwards Life Sciences Germany | T440020A | |

## Appendix B: Substance P liquid phase extraction procedure (Yanaihara)[191]

1. 1 ml cold acetone is slowly added to 0.5 ml sample while vortexing to obtain a white precipitate. When not being handled, all tubes are kept capped to minimise evaporative losses.

2. Samples are centrifuged at 800G and 4°C for 10 minutes.

3. Supernatant is decanted and precipitate discarded.

4. 2 ml petroleum ether 40-60°C is added to supernatant.

5. Tubes are recapped and vortex mixed.

6. Samples are then centrifuged again at 800G and 4°C for 10 minutes.

7. Upper ether layer is discarded.

8. Lower acetone layer is dried under a stream of nitrogen at room temperature and stored at -20°C until radioimmunoassay.

9. Extracts are resuspended in 250 µl assay buffer solution just prior to radioimmunoassay.

**Appendix C: Solid phase extraction procedure: Mobile phase according to Euro-Diagnostica**[223]

1. Thaw the samples immediately before starting the extraction. Store at 2-8°C until adding 1M HCl.

2. The column is wetted with 5 ml methanol.

3. Wash the column with 20 ml distilled water.

4. Apply 2 ml plasma sample to which has been added 0.2 ml 1M HCl on the column. The flowrate should not exceed 2 ml per minute.

5. Wash with 20 ml 4% acetic acid in distilled water.

6. Elute the peptide with 4 ml methanol and collect the eluate in a 10 ml conic glass tube.

7. Evaporate the methanol in a stream of air or nitrogen.

**Appendix D: Solid phase extraction procedure: Mobile phase according to Fehder[224]**

1. Equilibrate columns with 5 ml methanol, followed by 5 ml distilled water.

2. Dilute sample 1:4 with 4% acetic acid (v/v) and vortex mix.

3. Load acidified sample onto column under gravity only.

4. Wash with 10 ml 4% acetic acid (v/v) under gentle vacuum.

5. Elute with 60% acetonitrile in 1% trifluoroacetic acid (v/v), prepared in distilled water. Use 1 ml of eluant, under gravity only.

6. Dry eluate under a stream of nitrogen at 45°C in a water bath.

**Appendix E: Solid phase extraction procedure: Mobile phase according to Peninsula (modified)**[225]

1. Acidify samples with equal volume of 1% TFA.

2. Centrifuge at 3900G and 4°C for 20 minutes.

3. Equilibrate IST Isolute C18 SPE 50mg/1ml columns with 1 ml 100% ACN followed by 9 ml 1% TFA.

4. Load sample supernatant onto column under gravity only.

4. Wash with 6 ml 1% TFA under gravity.

5. Elute with 1 ml of 60% ACN in 1% TFA under gravity only and collect eluant in a polypropylene tube.

6. Dry under a stream of nitrogen at 45°C in water bath.

TFA = trifluoroacetic acid
ACN = acetonitrile
SPE = solid phase extraction

1% TFA
- Dilute 1 ml concentrated TFA slowly in 99 ml distilled wataer

60% ACN in 1% TFA
- Dilute 0.4 ml concentrated TFA slowly in 39.6 ml distilled water
- Top up with 60 ml ACN to 100 ml

**Appendix F**

Substance P Radioimmunoassay Protocol

1. Set up SP standards in *polypropylene* tubes. Top standard is 500 pmol/L. Make up with 50 µl working stock SP (50 nmol/L) into 4950 µl phosphate buffer. Serially dilute 1:1 with more buffer to lowest standard 0.98 pmol/L.

2. Work out volume of SP antibody required (100 µl per assay tube). Dilute stock antibody 1:400 with buffer to final concentration of 1:120000.

**NB: Each 100 µl aliquot of SP stock antibody is sufficient for 400 mini assay tubes**

3. Label assay tubes as follows:

| | |
|---|---|
| 1 – 2 | Totals |
| 3 – 4 | NSB |
| 5 – 6 | Reference (0 pmol/L) |
| 7 – 8 | 0.98 pmol/L |
| 9 – 10 | 1.95 pmol/L |
| 11 – 12 | 3.9 pmol/L |
| 13 – 14 | 7.8 pmol/L |
| 15 – 16 | 15.6 pmol/L |
| 17 – 18 | 31.2 pmol/L |
| 19 – 20 | 62.5 pmol/L |
| 21 – 22 | 125 pmol/L |
| 23 – 24 | 250 pmol/L |
| 25 – 26 | 500 pmol/L |
| 27 – x | Samples in duplicate |

4. Pipette 50 µl buffer into NSB and Reference tubes. Pipette 50 µl standards into respective assay tubes 7 – 26. Pipette 50 µl reconstituted sample extracts in duplicate into relevant tubes. Totals contain only tracer.

5. Using repeater, pipette 100 µl SP antibody into each assay tube except Totals and NSB. Vortex mix all tubes and incubate at 4°C for 24 hours.

6. Work out volume of SP tracer required (100 µl per assay tube). Dilute stock tracer 1:40 with buffer.

7. Pipette 100 µl SP tracer into each assay tube. Vortex mix all tubes and incubate at 4°C for 24 hours.

8. Mix Sac-Cel well for 5 minutes. Using repeater, pipette 50 µl Sac-Cel into each assay tube except Totals. Vortex mix all tubes and incubate at 4°C for 1 hour.

9. Centrifuge at 1700G and 4°C for 15 minutes (Program 15).

10. Decant supernatants and count pellets in gamma counter on Program 54.

Neurokinin A Radioimmunoassay Protocol

1. Set up NKA standards in *polypropylene* tubes. Top standard is 1000 pmol/L. Make up with 50 µl working stock NKA (1 nmol/L) into 4950 µl phosphate buffer. Serially dilute 1:1 with more buffer to lowest standard 1.95 pmol/L.

2. Work out volume of NKA antibody required (50 µl per assay tube). Dilute stock antibody 1:75 with buffer.

**NB: Each 100 µl aliquot of NKA stock antibody is sufficient for 150 assay tubes**

3. Label assay tubes as follows:

| | |
|---|---|
| 1 – 2 | Totals |
| 3 – 4 | NSB |
| 5 – 6 | Reference (0 pmol/L) |
| 7 – 8 | 1.95 pmol/L |
| 9 – 10 | 3.9 pmol/L |
| 11 – 12 | 7.8 pmol/L |
| 13 – 14 | 15.6 pmol/L |
| 15 – 16 | 31.2 pmol/L |
| 17 – 18 | 62.5 pmol/L |
| 19 – 20 | 125 pmol/L |
| 21 – 22 | 250 pmol/L |
| 23 – 24 | 500 pmol/L |
| 25 – 26 | 1000 pmol/L |
| 27 – x | Samples in duplicate |

4. Pipette 50 µl buffer into NSB and Reference tubes. Pipette 50 µl standards into respective assay tubes 7 – 26. Pipette 50 µl reconstituted sample extracts in duplicate into relevant tubes. Totals contain only tracer.

5. Using repeater, pipette 50 µl NKA antibody into each assay tube except Totals and NSB. Vortex mix all tubes and incubate at 4°C for 24 hours.

6. Work out volume of NKA tracer required (50 µl per assay tube). Dilute stock tracer 1:60 with buffer.

7. Pipette 50 µl NKA tracer into each assay tube. Vortex mix all tubes and incubate at 4°C for 24 hours.

8. Mix Sac-Cel well for 5 minutes. Using repeater, pipette 50 µl Sac-Cel into each assay tube except Totals. Vortex mix all tubes and incubate at 4°C for 1 hour.

9. Centrifuge at 1700G and 4°C for 15 minutes (Program 15).

10. Decant supernatants and count pellets in gamma counter on Program 7.

**Appendix G**

## Study Collection Procedure

THERE ARE SPECIAL SALIVA AND BLOOD TUBES FOR THIS STUDY.

1. Check on babies and nursing staff quickly
   a) *Must* be >½ hour since any feeds
   b) *Note general condition and write down times of last handling and last feed.* Fill in a Sample Collection Details form

2. Perform saliva suction at –15 mmHg with trap (maximum *10 minutes* duration).

3. Cap saliva trap immediately and place in –20°C freezer. *Note time of end of suction.*

4. Perform venepuncture, collecting 0.75 – 1 ml into white microtube. Keep on ice when not being handled. *Note time of sampling.*

5. Spin white microtube in microfuge for 1 minute. Remember to use the appropriate green balance.

6. Aliquot plasma
   - only 3 drops into **yellow** microtube
   - rest of plasma into **pink** microtube

7. Mix pink tube well so preservative dissolves and place in –20°C freezer. Time between blood collection and plasma freezing should be 10 minutes or less.

**Appendix H**

# INFORMATION FOR PARENTS

## An investigation into the measurement of pain and stress in newborn infants

Babies cannot easily tell us when they are comfortable or when they are in pain. Because we look after a lot of ill babies we would like to learn reliable ways of deciding how comfortable they are. We are performing a study in the neonatal unit to try and find out more about this, to help us improve the way that we can look after babies in the future.

We know that when adults are in pain their bodies produce chemicals called substance-P, neurokinin-A and cortisol that circulate in their blood, but we do not know if this occurs in newborn babies. These chemicals can be measured using a small blood sample. We are trying to find out if the same measurements can be useful in newborn babies and if these chemicals are also present in the saliva as well as the blood. To do this we would like to ask your permission to collect a small sample of blood (0.5ml) and a small sample of saliva (0.25ml) from your baby. The blood sample would be collected at a time when your baby's doctor was doing a blood test anyway so there would not be any extra needle-punctures required for the study. The saliva would be collected by gently sucking it into a small tube placed in your baby's mouth. Neither the blood sample nor the saliva sample would be stressful or harmful to your baby.

If you allow your baby to be included in this study it may help us understand more about how to assess pain in newborn babies, but the study will be of no direct benefit to your child. The study does not involve any treatments and your baby will be cared for in exactly the same way whether or not you choose to give us your permission. If you give us your permission, you will be free to change your mind at any time. If you would like any more information about this study please feel free to ask at any time.

*For further information, please ask for:*

Dr Mae Wong
Research Fellow in Neonatology
Ext 64350/64525

# LOTHIAN RESEARCH ETHICS COMMITTEE

# STANDARD CONSENT FORM

TITLE OF THE PROPOSED RESEARCH:

**An investigation into the measurement of pain and stress in newborn infants**

NAME OF INVESTIGATORS:

**Dr Mae Wong, Dr Ben Stenson, Dr Ian Laing, Dr Andy Lyon, Professor Neil McIntosh**

ADDRESS:

**Neonatal Unit, Simpson Memorial Maternity Pavilion, Edinburgh**

TELEPHONE:

**0131-5364350**

FURTHER INFORMATION IS AVAILABLE FROM: (A person who is not involved in the study) **Dr Paula Midgley**

- ☐ **I agree to my child participating in this study.**
- ☐ **I have read this consent form and Parent Information Sheet and had the opportunity to ask questions about them.**
- ☐ **I agree for notice to be sent to my child's General Practitioner about their participation in this study.**
- ☐ **I agree to the provision of any clinically significant information to my child's General Practitioner.**
- ☐ **I understand that my child is under no obligation to take part in this study and that a decision not to participate will not alter the treatment that my child would normally receive.**
- ☐ **I understand that my child has the right to withdraw from this study at any stage and that to do so will not affect their treatment.**
- ☐ **I understand that this is non-therapeutic research from which my child cannot expect to derive any benefit.**

**Name of Patient:**.....

**Signature of Parent or Guardian**.....

**Signature of Investigator:** .....

**Date**.....

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# Publications

Pediatric Research 54(4):574, 2003



# 0246NEO

## THE EFFECT OF PAIN ON PLASMA SUBSTANCE P & NEUROKININ A IN NEONATES

C Mae Wong, Elaine M Boyle, Ian A Laing, Rhona I Stephen, John Smith, Neil McIntosh
Simpson Centre for Reproductive Health, Royal Infirmary of Edinburgh City: Edinburgh, UK

**Background:** Substance P (SP) and neurokinin A (NKA) are neuropeptides involved in pain sensory signal processing[1]. They can be measured in plasma and have been associated with pain and inflammation in adults[2,3]. There is a possible role for them as markers of persistent pain in neonates, but this has not previously been investigated. The aim was to determine whether plasma SP and NKA levels varied with pain. **Methods:** This was a prospective case-controlled observational study. From July 2000 to December 2001, blood samples were collected longitudinally from 19 neonates believed to be suffering pain, e.g. due to surgery, necrotising enterocolitis, severe intraventricular haemorrhage, and meningitis. 19 controls matched for gestation and ventilatory status were recruited. Gestation ranged from 25–41 weeks. Only one sample per infant per day was taken between 0800 and 1200 hours. Neuropeptides were extracted, then measured using an in-house radioimmunoassay. Results were analysed using the Mann-Whitney U test. **Results:** Area under the curve (AUC) was calculated for three consecutive daily neuropeptide levels. There was no significant difference in median AUC for either SP (pain=3.79, control=3.03, P=0.8) or NKA (pain=10.2, control=12.3, P=0.3). One limitation of this observational study was that 15 of 19 infants believed to be suffering pain already received analgesia prior to study enrollment. Analysis of the 4 who had not received prior analgesia yielded no significant differences compared with either those who had received analgesia, or the controls. Graphs of peptide levels over time were also examined for individual infants and no consistent change noted with pain or analgesia administration. **Conclusion:** This is the first study of SP and NKA levels in neonates. Pain does not appear to have a significant effect on levels of either neuropeptide. This may be due to adequate treatment of pain with analgesia administration. It is also possible that some or all of the infants were not in pain. Only 4 did not receive analgesia. The appropriate treatment of pain should not be withheld for any study. Therefore, it may be more feasible to study SP and NKA levels in a population of infants who are not routinely receiving analgesia, but exposed to another source of possible pain or distress, such as assisted ventilation. 1. Fleetwood-Walker S. Current Opinion in Anaesthesiology 2:645–648, 1989. 2. Marshall KW. Arthritis and Rheumatism 33(1):87–90, 1990. 3. Gallai V. Cephalalgia 15(5):384–390, 1995.

# 0249NEO

## SUBSTANCE P & NEUROKININ A IN NEONATES: EFFECT OF VENTILATION & ANALGESIA

C Mae Wong, Elaine M Boyle, Ian A Laing, Rhona I Stephen, John Smith, Neil McIntosh
Simpson Centre for Reproductive Health, Royal Infirmary of Edinburgh City: Edinburgh, UK

**Background:** Substance P (SP) and neurokinin A (NKA) are neuropeptides involved in transmission and modulation of pain signals[1]. They are measurable in various body fluids and have been associated with pain and inflammation in adults. They could potentially be neurochemical markers of pain in neonates. The aims of this study were to investigate the effect of ventilation on SP and NKA levels in preterm infants, and to see if analgesia affected these levels. **Methods:** From July 2000 to December 2001, blood samples were collected longitudinally from 142 neonates, gestation 23–42 weeks. Only one sample per infant per day was taken between 0800 and 1200 hours. Plasma was extracted and neuropeptides measured using an in-house radioimmunoassay. Infants with presumed painful conditions were excluded. Results were analysed using the Mann-Whitney U test. **Results:** Median neuropeptide values (pmol/L) are tabulated. Table 1a shows SP results from all neonates grouped by ventilation, and Table 1b shows corresponding NKA results. SP levels were not found to be significantly higher in ventilated infants. NKA levels were significantly lower in ventilated infants on days 1 & 2, and higher on days 7 & 14. Of 142 neonates, 33 infants ≤32 weeks' gestation were also enrolled into a randomised double-blinded controlled trial investigating the routine use of morphine infusions to sedate ventilated preterm infants. All infants on a study drug infusion (21 morphine, 12 placebo) were ventilated. Gestation, birth weight and antenatal factors were similar in both groups. Area under the curve (AUC) was calculated for neuropeptide levels on days 1 to 3. There was no significant difference in median AUC for SP (morphine=3.21, placebo=3.38, P=0.5). Median AUC for NKA was significantly lower in those ventilated infants who received morphine (morphine=11.4, placebo=13.0, P=0.05). **Conclusion:** This is the first study to investigate changes in SP and NKA levels with ventilation. SP levels do not appear to be a useful marker of persistent pain or distress. Conversely, NKA levels show significant changes with ventilation, and are further modulated by the use of analgesia. We previously reported cortisol responses in the same group of infants, demonstrating significant changes with ventilation but not with analgesia[2]. It appears that although cortisol is a useful indicator of overall stress, NKA might be more specific for pain.

Table 1a

| Postnatal day | Ventilated | | Not Ventilated | | P value |
|---|---|---|---|---|---|
| | *N* | *SP* | *N* | *SP* | |
| 1 | 39 | 1.10 | 31 | 1.47 | 0.7 |
| 2 | 40 | 1.63 | 36 | 1.34 | 0.6 |
| 3 | 46 | 2.03 | 96 | 2.00 | 0.2 |
| 7 | 30 | 1.89 | 85 | 1.75 | 0.4 |
| 14 | 20 | 1.51 | 62 | 1.33 | 0.5 |

Table 1b

| Postnatal day | Ventilated | | Not ventilated | | P value |
|---|---|---|---|---|---|
| | *N* | *NKA* | *N* | *NKA* | |
| 1 | 39 | 5.64 | 31 | 6.82 | 0.01 |
| 2 | 40 | 5.85 | 36 | 7.06 | 0.01 |
| 3 | 46 | 6.20 | 96 | 6.67 | 0.2 |
| 7 | 30 | 6.12 | 85 | 5.26 | 0.02 |
| 14 | 20 | 5.40 | 62 | 4.64 | 0.01 |

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# 0250NEO

## IN-LINE PRESSURE MEASUREMENTS IN A MODEL OF 3-COMPONENT I.V. THERAPY.

[1]R Gelissen, [1]R van Rens,[2]M Schasfoort, [2]H vd Waterbeemd, [3]R Berendsen, [1]P Andriessen
Máxima Medical Center, [1]NICU, [2]Medical Technology, [3]Depart of Clinical Physics, City: Veldhoven, The Netherlands (sponsor CE Blanco)

**Aim:** We investigated (in-line) pressure course during and after occlusion of different pumps in a simulation model in order to choose an appropriate pump system for our (pre)term infants. **Methods:** 5 different (syringe) pumps were tested in the study. The model simulated i.v. therapy of 3 components, comparable to T.P.N. administration used in a 1-kg preterm infant. The pumps were positioned vertically: Upper pump (UP) with a flow rate of 2,5 ml/h, middle pump (MP) with a flow rate of 1,6 ml/h and the lower pump (LP) with a flow rate of 0,3 ml/h. In-line pressure was measured using a DTX™ Plus Transducer (sampling rate of 1 Hz) at UP, MP, LP and just before the location of the IV administration. Measurements per model in triplicate: Δt1 time delay between start of infusion therapy and first droplet was used as an estimate of "being on pressure" of the system, Δt2 time delay between occlusion and first alarm, Δt3 time delay between discontinuing the occluding pump and second alarm, ΔP automatic pressure decline after attaining the pressure limit was used as an estimate for in-line mixing of components. Data expressed as mean (SD).

**Results:**

| Results: | | | | |
|---|---|---|---|---|
| Pump | Δt1 (s) | Δt2 (min) | Δt3 (min) | ΔP (mmHg) |
| Terumo, Terufusion | 443 (49) | 17 (3) | - | - |
| Fresenius, Orchestra | 23 (2) | 21 (3) | 8 (2) | 97 (19) |
| Braun, Perfusor FM | 36 (2) | 9 (0) | 3 (0) | 36 (3) |
| Graseby, Omnifuse | 32 (4) | 22 (1) | 14 (0) | 114 (1) |
| Alaris, Atom CC | 38 (5) | 17 (1) | 1 (1) | 35 (7) |

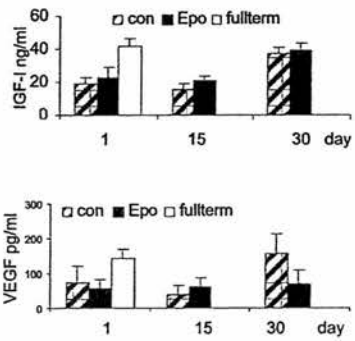
**Conclusion:** Considerable differences exist in (in-line) pressure course and alarm function between the pumps. 4 out of 5 are on pressure within 1 min. Braun and Alaris react quicker after occlusion. In-line mixing between the different components was remarkably less with Braun and Alaris.

# 0254NEO

## LEVELS OF IGF-I VEGF IN RHUEPO TREATED PREMATURES FOR ANEMIA OF PREMATURITY (AOP): ASSOCIATION WITH RETINOPATHY OF PREMATURITY (ROP), (PILOT STUDY).

Soubasi V, Diamanti E, Vyzantiadis T, Spandou E, Laporda A, Tsantali Ch, Kremenopoulos G.
Depts of Neonatology, Physiology, Lab of the 2nd Prop Dept of Internal Medicine,Aristotle University of Thessaloniki. City: Thessaloniki, Greece

**Background:** Based on experimental data on adult murine retinas, systemic application of rHuEpo crosses the blood-retina barrier. Also, when it was administered before or immediately after retinal ischemia, not only reduced histopathological damage but promoted functional recovery (Grimm et al Nat Med 2002). Besides, VEGF is instrumental in the development of abnormal retinal vasculature and IGF -1 is necessary at minimal levels to promote maximum function of VEGF. When IGF-I is persistently low, vessels cease to grow and VEGF accumulates in the vitreous, leading subsequently in neovascularization and ROP. Erythropoietin (Epo) interacts with VEGF to potentiate its vascular activity. Epo and VEGF often co-distribute and are regulated by hypoxia inducible factor (HIF-I).OBJECTIVE: The aim of this prospective controlled study was to assess, if early rHuEPO treatment for AOP would have a preventive effect in ROP through optimizing both VEGF and IGF-I. **Methods:** 15 premature neonates (GA<30 wks, BW<1250g) were entered in a randomized controlled study of rHuEPO treatment. The neonates assigned to receive either rHuEPO (250 units/g/every other day) or not, early after birth. Levels of VEGF and IGF-I were measured in both serum and plasma at 1st ,12–16th and 30–35th day after birth using enzyme immunoassay and immunoradiometric assay respectively. Clinical and ROP data were recorded. VEGF and IGF-I levels were also measured in 12 fullterm neonates the 1st day of life.



Early Human Development 74:71, 2003



in the duration of insult which is required to produce an isoelectric EEG [2], and human babies are not subjected to identical and reproducible insults. Despite this, we felt that it would be interesting to examine the relationship between the EEG determination of seizure onset time and our best estimate of the time of any fetal/neonatal insult in the human body.

**Aims:** To establish whether or not the time of seizure onset, as determined by EEG monitoring, was related to the time of the fetal/neonatal cerebral insult [3]. Previous work has examined the time of clinical seizure onset, which is unreliable when compared to EEG.

**Methods:** Babies who were considered to be high risk of seizure were monitored prospectively using 12-channel continuous video-EEG telemetry, with parental consent. The best estimate of the time of brain injury was determined by careful review of the CTG traces, the obstetric history, and the peripartum events by an observer (AD) who was blind to the EEG findings. In one case, there were neuropathological findings from autopsy.

**Results:** Eight babies with hypoxic–ischaemic encephalopathy had monitoring commenced sufficiently early to allow detection of the time of first electrographic seizure, and a further baby was already seizing when monitoring began (at 5 h). In three babies, it was thought that the insult had occurred before labour and more than 12 h before birth. In this group, the EEG seizure onset time was 4, 5.5, and 9.5 h, respectively. These babies began seizing earlier than the six whose insult was thought to have occurred during labour or close to the time of birth. In these babies, the first seizure was detected at 11.5, 12, 13, 18, 20, and 26 h after birth. This gave a median seizure onset time of 15.5 h compared to 5.5 h (Mann–Whitney $p$-value = 0.02).

**Conclusion:** Babies who had suffered an acute hypoxic–ischaemic insult close to birth had their first EEG seizure later than those whose history suggested that their brain injury was sustained before labour began. The EEG in this group followed a very similar pattern to that seen in the lamb model after an acute insult. Our results suggest that babies with EEG seizure onset at less than 6 h probably did not sustain their brain injury at around the time of birth. Continuous EEG monitoring from birth may allow a more precise estimate of the time of brain injury to be made than that which is currently possible with other methods.

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## Plasma substance P and neurokinin A in neonates—variation with gestation and postnatal age

C. Mae Wong, Elaine M. Boyle, Ian A. Laing, Rhona I. Stephen, John Smith, Neil McIntosh

Neonatal Unit, Simpson Centre for Reproductive Health, Royal Infirmary of Edinburgh, Edinburgh EH16 4SA, UK

**Background:** Substance P (SP) and neurokinin A (NKA) are neuropeptides involved in transmission and modulation of pain signals [1]. They are measurable in plasma and have been associated with pain in adults [2,3]. There is a potential role for them as neurochemical markers of pain in neonates, but this has been previously investigated.

**Aim:** As a baseline to studying plasma SP and NKA as possible markers of pain in neonates, the effect of gestation and postnatal age was studied to establish 'normal' values.

**Methods:** From July 2000 to December 2001, longitudinal once-daily morning blood samples were collected on days 1, 2, 3, 7, and 14 neonates, gestation 23–40 weeks. Peptides were extracted, then quantified using an in-house radioimmunoassay. Infants with presumed painful conditions were excluded.

**Results:** Overall, SP levels ranged from 0.0 to 11.2 pmol/l (median 1.7 pmol/l) and NKA levels from 1.8 to 74.6 pmol/l (median 6.0 pmol/l). Gestation and birth weight had no significant correlation with peptide levels. Postnatally, there was a gradual rise in median SP during the first 3 days which decreased again by day 14 (median NKA showed a similar rise over the first 3 days but decreased by day 7). These patterns were more apparent in preterm infants $\leq 32$ weeks' gestation.

**Conclusions:** This is the first description of normative values of SP and NKA in neonates. SP and NKA show changes with postnatal age, more marked in preterm infants. In early postnatal days, multiple factors may influence SP and NKA levels, including antenatal circumstances, ventilation, parental nutrition, and drugs. Further elucidation of these factors is warranted.

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## The predictive value of EEG monitoring for subsequent intracerebral complications during ventilation in preterm infants

Henning Wulbrand

The Eppendorf Center for Pediatric Neurology, Hamburg, Germany

**Objectives:** Only little data exist on bioelectrical activity of the brain in preterm infants during the periods of increased risk of intracerebral impairment during the first days of life.

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**Purpose of the study:** To evaluate the quality of care delivered by Advanced Neonatal Nurse Practitioners at Ashington Hospital.

**Methods:** A novel methodology for confidential enquiry was developed, which allowed aspects of high-quality practice to be captured as well as suboptimal care. Sentinel cases included all babies who, within the first postnatal week, either died, transferred out, or developed convulsions. Five other hospitals, all with different models of neonatal care, but all based on hierarchies of medical staff, were chosen as comparators. Eight dimensions of care were each rated on a scale of 0 to 4 (from seriously deficient to exemplary). Case notes for babies born between April 1998 and March 1999 were anonymised and assessed blind by four raters. If predefined standards of concordance were not met, the notes were rated by a panel independent of any of the participating hospitals, and this rating was taken as definitive. Arithmetical means of the scores were taken for each relevant dimension of care for each case, and the mean for each case was averaged for each hospital.

**Results:** Eighty-two cases were analysed. The dimension relating to terminal care had insufficient numbers for inclusion. The lowest score for any single case was 0.49 and the highest was 3.07; neither case was from Ashington. Mean scores for the comparator hospitals were 1.80, 2.13, 2.32, 2.23 and 2.43. The mean for Ashington was 2.35. Results for each dimension for Ashington are related to the means for all the comparator hospitals in the table below. Ashington scored above average on six out of the seven dimensions.

| | Comparator Average | Ashington |
|---|---|---|
| Resuscitation | 2.33 | 2.59 |
| Admission | 2.27 | 2.37 |
| Stabilisation | 2.14 | 2.45 |
| Investigations | 2.39 | 2.25 |
| Communication | 1.84 | 2.07 |
| Treatment | 2.16 | 2.5 |
| Transfer out | 2.07 | 2.21 |

**Conclusions:** The quality of care of these sentinel cases appeared to be at least as good when provided by Advanced Neonatal Nurse Practitioners alone as when delivered by medical models of neonatal care. These data support the contention that good quality neonatal care can be delivered by Advanced Neonatal Nurse Practitioners alone and does not require the presence of resident junior paediatricians.

### Changes in plasma cortisol with ventilation and analgesia in preterm infants

C Mae Wong, Elaine M Boyle, Ian A Laing, Joan Yorke, Jaqi Armstrong, John Smith, Paula C Midgley, Neil McIntosh

Neonatal Unit, Simpson Centre for Reproductive Health, Royal Infirmary of Edinburgh, Edinburgh EH16 4SA, UK

**Background:** Plasma cortisol can be used as a marker of pain and stress in neonates[1]. Preterm infants often require assisted ventilation, but analgesia is not used routinely.

**Aims:** To investigate the effect of ventilation on plasma cortisol levels in preterm infants, and to see if analgesia affects these levels.

**Methods:** From July 2000 to December 2001, longitudinal once-daily morning blood samples were collected from 88 neonates $\leq 32$ weeks gestation. Plasma cortisol was measured by a direct in-house radioimmunoassay. Infants with painful conditions were excluded. No infant received postnatal steroids. Results were analysed by Mann–Whitney *U*-test.

**Results:** Median cortisol values (nmol/l) are tabulated. Table 1 shows results from the 88 neonates grouped by ventilation. All the ventilated babies are shown in Table 2 grouped by analgesia. Within this ventilated cohort was a subgroup of infants randomised in the NEOPAIN Trial to morphine or placebo infusions. Samples taken during assisted ventilation showed significantly higher cortisol levels. However, there was no significant difference in cortisol levels between the groups receiving analgesia or no analgesia. Similar results were obtained with samples from the subgroup of NEOPAIN infants. There was no difference in gestation or birth weight between those who received analgesia and those who did not.

Table 1

| Postnatal day | Ventilated | | Not Ventilated | | *P* value |
|---|---|---|---|---|---|
| | *N* | Cortisol | *N* | Cortisol | |
| 1 | 34 | 309.7 | 18 | 99.5 | 0.006 |
| 2 | 35 | 310.0 | 17 | 107.8 | 0.001 |
| 3 | 36 | 209.1 | 39 | 133.3 | 0.002 |
| 7 | 26 | 273.1 | 45 | 176.9 | 0.009 |
| 14 | 19 | 211.6 | 38 | 147.7 | 0.05 |

Table 2

| Postnatal day | Morphine | | No morphine | | *P* value |
|---|---|---|---|---|---|
| | *N* | Cortisol | *N* | Cortisol | |
| 1 | 19 | 351.1 | 15 | 199.1 | 0.19 |
| 2 | 17 | 292.4 | 18 | 356.1 | 0.30 |
| 3 | 17 | 207.3 | 19 | 286.8 | 0.68 |
| 7 | 6 | 350.1 | 20 | 273.1 | 0.93 |
| 14 | 4 | 207.6 | 15 | 211.7 | 1.00 |

**Conclusions:** Assisted ventilation is associated with raised plasma cortisol levels in preterm infants. This could be due to the underlying cause of the need for ventilation or the pain or stress of ventilation itself. However, analgesia has no effect on these levels in ventilated infants, suggesting either that morphine is ineffective in ameliorating pain or that the stress of ventilation is not related to pain.

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Pediatric Research 52(5):787, 2002



## 47

**EFFECTS OF OXYGEN TENSION (OT), CORTICOSTERONE (CST) AND CORTICOTROPIN-RELEASING-HORMONE (CRH) ON NA+ TRANSPORT IN RAT FETAL ALVEOLAR CELLS**

Ulrich H. Thome, Ian C. Davis, Susie V. Nguyen, Janice Ware, Brent J. Shelton, and Sadis Matalon, University of Alabama at Birmingham, Birmingham AL, USA

Background: Oxygen and steroids are involved in mediating the transition from fluid secretion to fluid absorption in the perinatal lung. CRH is thought to control the timing of birth in several species.

Aims: To investigate how OT, CST and CRH affect active Na+ transport by fetal distal lung epithelia (FDLE) isolated from 19 d rat fetuses (term: 22d).

Methods: FDLE were grown to confluent monolayers on permeable supports in 5, 12 or 20% O2 with 5% CO2, using serum free media supplemented with 1 μmol/l CST and/or 3 nM CRH. Short circuit currents (ISC) were measured in Ussing chambers. Baseline ISC (BS), generated by intact cells, ouabain sensitive ISC (OS) and amiloride sensitive ISC (AS) were measured. Expres-sion of Na+ transport proteins was determined by SDS-PAGE and Western blot.

Results: Data from 250 monolayers, 4-11 per condition, were analyzed with 3-way ANOVA. With increasing OT during culture, baseline ISC (BS) increased from 5.3±0.2 μA/cm2 at 5%, to 8.4±0.3 μA/cm2 at 20% (p<0.001). OS increased from 3.4±0.6 to 9.1±0.6 μA/cm2 (p<0.001), and AS from 3.4±0.2 to 4.3±0.2 μA/cm2 (p<0.01). CST did not change BS or OS, but increased AS from 3.1±0.2 to 4.6±0.2 μA/cm2 (p<0.001). CRH did not have an effect on ISC. Protein expression was analyzed in cells derived from 4 different isolations. Culture under 5% O2 in the presence of CST significantly reduced expression of a-, b-, and g-ENaC and a1-Na,K-ATPase subunits, but did not change the b1-Na,K-ATPase subunit. Expression of these proteins was similar in cultures grown under 20% O2 with or without CST, or 5% O2 without CST.

Conclusion: Increased OT exposure resulted in increased basolateral Na,K-ATPase function as well as apical Na+ permeability, whereas CST only increased apical Na+ permeability, thus limiting the effect of CST on maximum Na+ transport. CRH did not affect Na+ transport. Changes in function did not correlate well with changes in transport protein expression.

## 48

**REACTIVE INTERMEDIATES OF SIN-1 MODULATE CHLORIDE SECRETION ACROSS MURINE AIRWAY CELLS**

Ulrich H. Thome, Ahmed Lazrak, Lan Chen, Michael J. Thomas (1), Henry Jay Forman and Sadis Matalon, University of Alabama at Birmingham and (1) Wake Forest University, Birmingham AL, USA and (1) Winston-Salem NC, USA

Background: Reactive oxygen/nitrogen species are generated in increased amounts during inflammatory processes and may affect epithelial ion transport.

Aims: To quantify the effects of reactive oxygen-nitrogen intermediates on chloride (Cl-) currents across cultured murine tracheal epithelial (MTE) cells.

Methods: Reactive species were generated by decomposition of 3-morpholinosydno-nimine (SIN-1). Water-tight MTE monolayers were grown on permeable supports. The effects of SIN-1 on short-circuit currents (ISC) were measured in Ussing chambers after completely blocking Na+ currents with amiloride (10μM). Single MTE cells were grown on glass coverslips, and the effects of SIN-1 on whole cell current and single Cl- channels were studied by the patch clamp method in whole-cell and cell-attached mode, using Na+-free solutions. Spectrophotometry and electrospray mass spectroscopy were used to identify new compounds.

Results: ISC increased after addition of SIN-1 (1 mM) from 7.6 ± 0.51 μA/cm2 to 35 ± 1.3 μA/cm2 (X ± 1 SE) within 30-60 min (EC50 = 7 μM), and returned to baseline values by 120 min. Forskolin (10 mM) augmented the SIN-1 effect when added early on, but failed to increase ISC when added 40-80 min post SIN-1. The ISC increase was blocked by glibenclamide (200 μM) or 5 mM reduced glutathione. In patch clamp experiments, SIN-1 temporarily increased whole cell Cl- current 4-fold, and open probability of single cystic fibrosis transmembrane regulator channels from 0.041% to 0.92%. Decomposed SIN-1 also increased ISC with an EC50 of 5 μM. Spectrophotometry and electrospray mass spectroscopy revealed the formation of several new compounds during the decomposition of SIN-1.

Conclusion: One or more of the metastable compounds formed during SIN-1 decomposition caused a transient increase, followed by a decrease, of epithelial Cl- transport.

## 49

**FATTY ACID STATUS IN BREASTFED AND FORMULA FED HYPERPHENYLALANINEMIC (HPA) INFANTS AT DIAGNOSIS.**

C Agostoni, E Riva, E Verduci, F Salvini, L Fiori, A Zenga, M Giovannini, Dept. of Pediatrics, San Paolo Hospital, Milan

**Objectives.** To compare biochemical parameters potentially associated with the later neurodevelopmental outcome (blood Phenylalanine, Phe, and fatty acids) between breastfed (BF) and formula-fed (FF) infants with HPA. **Study design.** In a cross-sectional study all HPA detected by newborn screening and referring to our Center in the last 2 years have been sampled for plasma Phe and fatty acids at entry and compared according to the early type of feeding. **Methods.** Blood Phe by amino acid analyser, FA by capillary gas-chromatography, statistics with Mann-Whitney non-parametric test. **Results.** Twenty-six subjects (17, BF, and 9, FF) were admitted. The median age for both groups at blood sampling was 18 days (ranges: 15-20 d for BF, 15-22 for FF). Plasma values (mean+SD) are reported in the Table.

| Parameter | BF (n=17) | FF (n=9) | P |
|---|---|---|---|
| Phe (umol/L) | 1177±987 | 1153±1104 | 0.98 |
| C18:2n-6 % | 20.8± 3.0 | 24.7±4.4 | 0.01 |
| C20:4n-6 % | 8.5 ± 2.3 | 5.4±1.5 | 0.001 |
| C18:3n-3 % | 0.25±0.11 | 0.51±0.22 | 0.003 |
| C20:5n-3 % | 0.21±0.06 | 0.18±0.07 | 0.10 |
| C22:6n-3 % | 2.1 ± 0.7 | 1.5 ± 0.5 | 0.06 |

**Conclusions.** Differences in the early LCPUFA more than Phe status could be involved in the differences found in the later outcome described in BF vs FF children with HPA, but a cause-effect relationship should be investigated in long-term follow-up studies.

**Conclusions.** Differences in the early LCPUFA more than Phe status could be involved in the differences found in the later outcome described in BF vs FF children with HPA, but a cause-effect relationship should be investigated in long-term follow-up studies.

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**INCIDENCE AND RISK FACTORS FOR HEARING LOSS IN DUTCH NICU-INFANTS**

ETM Hille[1], HLM van Straaten[2], PH Verkerk[1], 1 TNO-PG, Leiden, 2 Isala Clinics, Zwolle, The Netherlands

*Aim:* To determine the incidence and risk factors for congenital hearing loss (CHL) in at risk newborns in the Netherlands. *Design:* Automated Auditory Brainstem Response (AABR) hearing screening has been introduced since 1998 in the Dutch Neonatal Intensive Care Units (NICU). The results of all NICU-graduates are registered in a central database to facilitate the services needed for screening and following up of all infants with abnormal screening results. All NICU-graduates with one or more risk factors according to the Joint Committee on Infant Hearing were included in a two stage program. After a second failure diagnostic ABR was used to establish diagnosis of CHL. Newborns who died before the age of 3 months were excluded. *Statistics:* Univariate analyses of risk factors for CHL were performed. *Results:* In December 2001 4562 newborns (born before 1st of April 2001), median age 32 weeks (24-43) and 1455g (500-4850), have been included. The overall incidence of uni- or bilateral CHL after diagnostic ABR was 132/4562 (2.9%). Table: Odds Ratios (OR; 95%CI) of risk factors for CHL

Table: Odds Ratios (OR; 95%CI) of risk factors for CHL

| | OR | 95%CI |
|---|---|---|
| Familial CHL | 3.5 | [1.6-7.8] |
| Craniofacial anomalies | 7.1 | [4.2-12.2] |
| Birth weight <1500g | 0.6 | [0.4-0.8] |
| Hyperbilirubinemia requiring exchange | 1.4 | [0.6-3.4] |
| Ototoxic medications | 1.3 | [0.8-2.4] |
| Cerebral complications | 2.1 | [1.4-3.2] |
| Low APGAR score | 1.2 | [0.8-1.7] |
| Mechanical ventilation ≥5 days | 1.6 | [1.2-2.4] |
| Syndromes associated with CHL | 5.5 | [3.2-9.2] |

*Conclusion:* AABR hearing screening in Dutch NICU-infants revealed an overall incidence of 2.9% CHL. Univariate analysis revealed that the following NICU-infants are at increased risk: infants with CHL in the family, craniofacial anomalies, a birth weight ≥1500g, cerebral complications, mechanical ventilation ≥5 days, and syndromes associated with CHL.

## 51

**INFLUENCE OF VENTILATION & ANALGESIA ON PLASMA CORTISOL IN PRETERM INFANTS**

C Mae Wong, Elaine M Boyle, Ian A Laing, Joan Yorke, Jaqi Armstrong, John Smith, Paula Midgley, Neil McIntosh, Simpson Centre for Reproductive Health, Edinburgh

Background: Plasma cortisol can be used as a marker for pain and stress in neonates. Preterm infants often require assisted ventilation but analgesia is not used routinely. The aims of this study were to investigate the effect of ventilation on plasma cortisol levels in preterm infants, and to see if analgesia affected these levels.

Methods: From July 2000 to December 2001, samples were collected longitudinally from 88 neonates ¡Ü32 weeks gestation. Only one sample per infant per day was taken between 0800 and 1200 hours. Plasma cortisol was measured by a direct in-house radioimmunoassay. Infants were excluded from analysis if they had a known painful condition at the time of sampling. No infant received postnatal steroids. 33 of the 88 infants were also enrolled into a randomised double-blinded controlled trial (RCT) investigating the routine use of morphine infusions to sedate ventilated preterm infants. All infants on a study drug infusion (21 morphine, 12 placebo) were ventilated. Gestation, birth weight and antenatal factors were similar in both groups. Results were analysed using the Mann-Whitney U test. Results: Median cortisol values (nmol/L) are tabulated. Table 1a shows results from the 88 neonates grouped by ventilation. Table 1b shows results from the subgroup of 33 RCT infants on a study drug infusion.

**Table 1a**

| Postnatal | Ventilated | | Not Ventilated | | P |
|---|---|---|---|---|---|
| day | N | Cortisol | N | Cortisol | value |
| 1 | 34 | 309.7 | 18 | 99.5 | 0.006 |
| 2 | 35 | 310.0 | 17 | 107.8 | 0.001 |
| 3 | 36 | 209.1 | 39 | 133.3 | 0.002 |
| 7 | 26 | 273.1 | 45 | 176.9 | 0.009 |
| 14 | 19 | 211.6 | 38 | 147.7 | 0.05 |

**Table 1b**

| Postnatal | Morphine | | Placebo | | P |
|---|---|---|---|---|---|
| day | N | Cortisol | N | Cortisol | value |
| 1 | 17 | 319.8 | 7 | 117.7 | 0.47 |
| 2 | 16 | 301.2 | 8 | 445.3 | 0.14 |
| 3 | 14 | 215.8 | 4 | 334.6 | 0.75 |
| 7 | 2 | 1268.9 | 4 | 195.6 | 1.00 |
| 14 | 0 | - | 4 | 186.6 | - |

Samples taken during assisted ventilation showed significantly higher cortisol levels. However, there was no significant difference between cortisol levels taken while on morphine versus placebo infusions. Conclusions: Ventilation is associated with raised plasma cortisol levels in preterm infants. This could be due to the underlying cause of the need for ventilation, or the pain or stress of ventilation itself. However, analgesia has no effect on these levels in ventilated infants. This suggests either that morphine is ineffective in ameliorating pain, or that the stress of ventilation is not related to pain.

## 52

**DIGOXIN-LIKE IMMUNOREACTIVE SUBSTANCE IN NONOLIGURIC HYPERKALEMIA**

Eva Mildenberger, Kathrin Oels, Karl Bauer, Martin Paul, Hans T. Versmold, Dept. of Pediatrics, Universitätsklinikum Benjamin Franklin, Berlin, Germany

Background / Aims: At around 24 h after birth very premature infants often present with an yet unexplained shift of potassium from the intra- into the extracellular space. This nonoliguric hyperkalemia probably results from a transient inhibition of the membrane-bound Na+/K+-ATPase. The serum of premature infants has endogenous digitalis-like activity, which inhibits the Na+/K+-ATPase as do digitalis glycosides. We hypothesized that this endogenous digitalis-like activity triggers hyperkalemia.

Methods: Serum concentrations of potassium ([K+]) and of the digoxin-like immunoreactive substance ([DLIS]) were measured during the first 24 h after birth in 60 infants including 30 infants < 30 gestational weeks (wks).

Results: [K+] at 24 h after birth was related to gestational age ($r^2$ = 0.48) and mean [K+] at 24 h after birth was higher in infants below 30 wks than in infants > 30 wks (5.7 +/- 1.0 (SD) (3.9 – 7.9) mmol/l vs. 4.3 +/- 0.6 (3.3 – 5.8) mmol/l, p < 0.0001). There was a negative linear correlation between [DLIS] at birth and [K+] at 24 h after birth ($r^2$ = 0.24). Contrary to our hypothesis, infants with lower [DLIS] at birth had higher [K+] at 24 h after birth. At 24 h after birth there was no correlation between [DLIS] and [K+]. At this time mean [DLIS] was similar in infants below and above 30 wks (0.5 +/- 0.3 (0 – 1.0) ng/ml vs. 0.6 +/- 0.2 (0.2 – 1.1) ng/ml, p = 0.07).

Conclusion: We doubt a major role of DLIS in nonoliguric hyperkalemia of the premature infant.